

# **Persistence of *Mycobacterium tuberculosis* in people without clinical disease**

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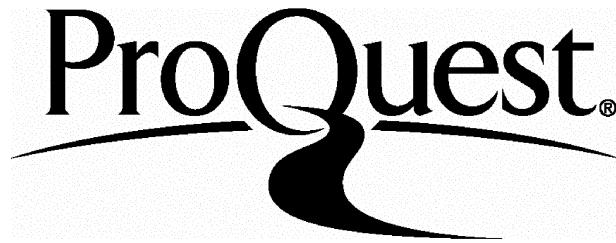
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**To**

**My parents**

## Abstract

*Mycobacterium tuberculosis* (MTB) can cause both active disease and latent tuberculosis (LTBI). Latent infection can reactivate and, is therefore, a potential reservoir of infection. Effective diagnosis and treatment of LTBI can only be achieved with a clear understanding of the pathogenesis of LTBI. This thesis attempts to develop an effective diagnostic method to detect LTBI and to clarify some aspects of its pathogenesis.

Tuberculin skin tests performed on Sri Lankan school children revealed a high prevalence of LTBI (40.5%) in Jaffna compared to Hambanthota (7.3%). Geographical variations and dietary habits of the population in the two locations have influenced reactions to tuberculin significantly.

Evaluation of antibody titre against 16kDa, 38kDa, 65kDa, & 70kDa antigens using ELISA revealed a significantly increased IgG response in elderly people. Titres to 16kDa and 38kDa antigens were as high as in people with active disease. However, isoelectric focusing revealed that the antibody response in elderly people was mainly oligoclonal to 16kDa whereas the oligoclonal response in active disease was mainly to the 38kDa antigen.

Polymerase chain reaction (PCR) detected MTB DNA in the sputum of 55% of elderly people, suggesting shedding of bacilli during LTBI. However, spoligotyping of the DNA ruled out transmission of infection within this community.

Detection of MTB DNA using PCR and *in situ* PCR on autopsy material of people without clinical TB suggests that MTB can lurk in arrested granulomas and in normal tissues during LTBI. Latency was maintained at various extrapulmonary sites including the brain. MTB DNA was detected in the cytoplasm, and less frequently inside the nucleus of non-professional phagocytic cells and in macrophages. Acid-fast and immunohistochemical staining failed to detect bacilli in sections adjacent to those that were MTB DNA positive. Spoligotyping of MTB DNA in these tissues revealed a conserved pattern.

In conclusion, this study has made some interesting findings, but raised many questions regarding the LTBI pathogenesis. Addressing these may help the development of effective diagnosis and treatment for LTBI.

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## Abbreviations

APC	Antigen presenting cells
ATP	Adenosin tri phosphate
BBB	Blood brain barrier
BSA	Bovine serum albumin
BCG	Bacillus Calmette Guerin
CD	Clusters of differentiation
CMI	Cell mediated immunity
CSF	Cerebrospinal fluid
CWD	Cell wall deficient
DR	Direct repeat
DAB	3, 3' diaminobenzidine
DNA	Deoxycarboxilic acid
DTH	Delayed type hypersensitivity
DVR	Direct variable repeat
DOTS	Directly observed treatment-short-course
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzymw-linkws immunospot
GusCN	Guanidiumthiocyanate
HBHA	Heparin-binding hemagglutinin
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
HSP	Heat shock protein
IEF	Isoelectric focusing
INH	Isoniazid
IFN- $\gamma$	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Ig	Immunoglobulin
IS-PCR	<i>In situ</i> polymerase reaction
LAM	Lipoarabinomannan
LTBI	Latent tuberculosis infection
MHC	Major histocompatibility complex
MMRc	Macrophage mannose receptor
MTB	<i>Mycobacterium tuberculosis</i>
MS	Multiple sclerosis
NADH	Nicotinamide-adenine dinucleotide (reduced)
NADPH	Nicotinamide-adenine dinucleotide phosphate (reduced)
NAF	Non-acid-fast
NCM	Nitrocellulose membrane
NO	Nitric oxide
NRP	Non-replicating
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate saline buffer
PCR	Polymerase chain reaction
PPD	Purified protein derivative
PZA	Prazinamide
RA	Rheumatoid arthritis

RFLP	Restriction fragment polymorphism
RIVM	National Institute of public health and environment
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
Rpf	Resuscitation promoting factor
RT-PCR	Reverse transcriptase Polymerase reaction
SDS	Sodium dodecyl sulphate
sTNFRII	Soluble type 2 tumour necrosis factor receptor
TB	Tuberculosis
TBM	Tuberculous meningitis
TCA	Tricarboxilic acid
TGF- $\beta$	Transforming growth factor beta
TNF- $\alpha$	Tumour necrosis factor alpha
TSB	Tris buffered saline
TST	Tuberculin skin test
WHO	World health organisation

# Chapter 1

## 1. Introduction

### 1.1 General introduction

Despite the longstanding effort to control tuberculosis (TB), it remains an expanding global health crisis, causing 3 million deaths and 8 million new cases annually (WHO, 1999). *Mycobacterium tuberculosis* (MTB), one of the etiologic agents of TB, is globally successful due to its ability to cause both an active disease as well as an asymptomatic latent infection, which is characterised by the persistence of low numbers of possibly non-replicating bacilli after initial infection (Das, *et al.*, 1993).

Infections due to MTB are commonly acquired via airborne transmission (Riley, *et al.*, 1995). The source of transmission is droplet nuclei, containing one to three bacteria, sufficient to cause infection, produced during coughing by persons suffering from pulmonary tuberculosis (Wiegeshaus, *et al.*, 1989). Inhaling droplet nuclei infects a susceptible person. Approximately 30% of exposed people become infected; 60-90% mount an immune response that causes the organism to enter into a latent phase where it can remain viable for many years (Snider and La Montagne, 1994). A small but significant percentage (~10%) of latent infections reactivate years to decades later to give rise to reactivation tuberculosis (Stead, 1967).

In the developed world reactivation of clinically latent infection is responsible for a large proportion of active tuberculosis despite claims that TB has been eradicated (Heldal, *et al.*, 2000, Martin and Lazarus, 2000). Latently infected people can develop clinical disease at any time. This may happen because of physical or emotional stress, old age, general ill health, or the development of immunodeficiency (Boomershine, 2000, Rajagopalan and Yoshikawa, 2000b, Waisser, *et al.*, 2000). Immune deficiency associated with conditions such as human immunodeficiency virus (HIV) infection, is a great risk factor of reactivation of latent infection (Murray, 1997). With the growing rate of HIV

infection in many underdeveloped and developing countries, the number of people infected with both TB and HIV represents a potential danger, as increasing numbers of actively infected hosts are capable of transmitting infection. Reactivation of latent infection has also been associated with ageing (Powell and Farer, 1980, Rajagopalan and Yoshikawa, 2000b, Rajagopalan and Yoshikawa, 2000a). The geriatric population represents the largest reservoir of MTB infection in developed nations, including the United States. Immigrants from high TB endemic areas can also act as reservoirs as there is high chance that these individuals are latently infected (Marks, *et al.*, 2000). In addition, poor socio-economic status of these people in the immigrant country contributes to the reactivation of latent infection and transmission of disease.

Current estimates are that one in three of the world's population is latently infected with MTB. They can be a reservoir of potential transmitters of the disease when it is reactivated. Therefore, effective TB eradication will only be possible if a clear understanding of the structural and functional biology of MTB and human immune response during latent infection is available. Knowledge of the persistence mechanism and the immunological responses evoked in the infected host may provide new strategies for preventing the establishment of latency and reactivation of latent infection.

**Definition of terminology:** As the physiological status of the tubercle bacilli during latent infection is not yet clear there are controversial views about the terminology used to describe the infection and the bacilli. Therefore, throughout this review, the following terms are used to avoid confusion:

1. **Persisters:** Organisms that survive antibacterial therapy
2. **Non-replicating bacilli:** Organisms that are prevented from growing by the immune system, but not killed by it.
3. **Latent infection:** This is the situation where there is evidence of past infection (usually based on the host immune response) but no evidence of active disease (paucibacillary).

4. Plateau phase of infection: Generally applied to animal models in which bacilli can be repeatedly grown from tissues (multibacillary), but there are no signs and symptoms.

## 1.2 History of tuberculosis

Tuberculosis has been recognised as an important, life-threatening human disease since the dawn of recorded history and pulmonary tuberculosis was first recognised during the classical Greek era (Hass, 1996). Archaeological evidence suggests that the source of tuberculosis was the Indo-Europeans known as Aryans. They shared language characteristics and migrated from the deciduous forests of central and eastern central Europe to the vast steppes north of the Black and Caspian seas and to the Indus valley about 1500 BCE, resulting in Hindu culture. References to tuberculosis can also be found in a number of ancient Hindu texts, in the Indian literature, including in the *Aharma-Veda* (Hass, 1996) where it is mentioned that

*The physician who values his reputation should not undertake to take care of a patient who has the three great symptoms: fever, cough, and bloody sputum. If, however, the patient has a good appetite and digests well the food, and the disease is in its infancy, a cure may be hoped for.*

TB has had many aliases throughout the history: The ancient Greeks called it *phthisis* (to waste). The TB of the skin was known as *lupus vulgaris* and bone TB as *Pott's* disease with characteristic vertebral fusion and deformity of the spine. The swollen gland of the neck (scrofula) was called The Kings Evil in Europe in mediaeval times because newly crowned kings of England and France were believed to have powers to heal TB with their touch. The most familiar term for tuberculosis at least to our grandparents and great-grandparent was *consumption* (to consume or wear away).

Exact pathology and anatomical description of the disease began to appear in the eighteenth century. In 1720, the English physician Benjamin Marten was the first to realise, that 'wonderfully minute living creatures' could cause TB, and to believe that consumption was contagious. In 1868, Villemin demonstrated the

contagious nature of consumption by inoculating rabbits with a mixture of grey and white tubercles obtained from lung and other organs of people suffering from consumption (Collins, 1998). His findings were almost uniformly ignored and the continued belief was that breathing unhealthy air caused consumption. Then came the breakthrough in uncloaking the causative agent of TB. In 1882, Robert Koch successfully cultured the tubercle bacillus on potato and inspissated serum slants in his laboratory. He discovered a staining technique that enabled him to visualise MTB. He also convincingly demonstrated it to be the cause of TB by injecting culture obtained from patients exhibiting the many clinical manifestations of this disease into rabbits and guinea pigs, which developed active tuberculosis.

Before Koch's revolutionary discovery, it was believed by the majority, that consumption was caused by stress in people with an inherited susceptibility. As there was no medicinal cure, Hippocrates recommended that a change of climate would improve the condition of consumptives. The first great intervention strategy known as the sanatorium movement began in Europe in the 1840s. George Bodington, a Warwickshire village practitioner was the first to advocate fresh outdoor air to consumptives. This was the beginning of the emphasis on the importance of healthy lifestyle (better housing, exercise and nutrition), which had steadily decreased the TB mortality rate. Edward Livingston Trudeau was the outstanding leader of the American sanatorium movement. With a simple experiment, exposing infected rabbits to various environmental conditions, Trudeau established that the progress of a tuberculous infection could be arrested, even reversed by a regimen of rest, good food, sunlight, and fresh air (Trudeau, 1887).

The sanatorium system did not significantly alter the mortality rate. At that time, mortality rate for consumption in the United States was around 500 cases per 100,000 persons per year, with survival rates ranging from 1 in 3 to 1 in 4 (Collins, 1998).

In the absence of any known cure, Koch tested a number of inactivated products prepared from tubercle bacilli to vaccinate people as Pasteur successfully did against rabies and fowl cholera. He used concentrated heat-inactivated culture

filtrate of tubercle bacilli (old tuberculin) to inject healthy and tuberculous guinea pigs. In healthy guinea pigs, old tuberculin did not cause any change. However, in tuberculous guinea pigs it caused necrosis at the site of inoculation as well as at the infection site: skin lesion. Infection sites on the skin underwent necrosis and fell off, resulting in cure of the skin TB. The reaction elicited at the site of the injection in the skin by the old tuberculin is referred to as Koch's phenomenon (Rook and al Attiyah, 1991). Thus, Koch found that old tuberculin would have some therapeutic benefit for the patient. Administration of old tuberculin cured TB in many cases but on the other hand, when it was given to patients with severe disease it resulted in fatal complications. This observation accords well with the theory that pre-existing immune response will influence the outcome of the second infection. Despite this early disappointment, old tuberculins still remains a useful diagnostic reagent.

Prophylactic intervention against TB started with the development of the *Bacillus of Calmette and Guerin (BCG)* vaccine. Calmette and Guerin developed a vaccine against TB by attenuating 'Lait Nocard', a strain of MTB isolated by Nocard from a cow that had tuberculous mastitis. They sub-cultured the organism once every three weeks on a glycerinated beef-bile-potato medium. In 1921, after 13 years and 230 subcultures, BCG had lost its virulence. Even though, BCG gives protection against TB in some populations (80% in U.K.) it has 0% effect in other populations such as South Indians (Stanford, 1983).

Active therapy began with the introduction of the artificial pneumothorax and surgical methods to reduce lung volume: MTB grows optimally in a well-oxygenated environment.

The end of the sanatorium era began with the discovery of a long awaited treatment method: chemotherapy. Even though antibiotic usage against infectious diseases had been underway for several years, they were ineffective against MTB. Discovery of streptomycin changed the situation. In November 1944, streptomycin was administered for the first time to a critically ill TB patient and the effect was almost immediately impressive. This brought the end to the widespread opinion that MTB was a bacteriological exception. A rapid succession

of anti-TB drugs appeared in the following years and combinations of two or three drugs were introduced to overcome the development of resistant mutants due to streptomycin monotherapy.

Commencing in the later half of the 20<sup>th</sup> century, the incidence of TB declined significantly in the industrialised nations and by the early 1980s it was widely believed that the disease had virtually been eradicated in developed countries. This complacent attitude was shattered in the early 1990s by the occurrence of an upsurge in the incidence of TB in New York City (Bloom and Murray, 1992). Due to the relentless spread of TB throughout the world, the World Health Organisation (WHO) in 1993 declared TB a global emergency (WHO, 1994). Furthermore, recently it has been reported that there was a 10% increase in the incidence of TB in greater London during last two years.

Resurgence of TB during the last two decades has been mainly due to the growing rate of HIV infection. Since HIV infection contributes to the immune deficiency of the host, reactivation of latent infection is common in this group. Recently, this has brought new attention to latent tuberculosis infection (LTBI). Nevertheless, LTBI has been recognised and researched since the early part of the last century. Live bacilli have been isolated from granulomas or tubercles as well as from macroscopically normal lung tissues from people with clinically inactive tuberculosis, indicating that the organism can lurk in the lung for many years (Opie and Aronson, 1927, Robertson, 1933). Part of the work described in this thesis provides further evidence of the presence of bacilli in macroscopically and microscopically normal lung tissue of 29% of Mexican autopsies obtained from people dying of causes other than TB (Hernandez-Pando, *et al.*, 2000). The purpose of this section is to review the current knowledge on latent tuberculosis infection.

## 1.3 Brief background

### 1.3.1 Human studies of latent infection

The knowledge of MTB during latency is limited. The mechanism of pathogenesis during reactivation of latent infection remains elusive. Currently available diagnostic methods such as bacteriological, serological, and molecular techniques are unable to effectively detect latent infection. Nevertheless, recently promising data of the diagnostic value of ESAT-6, an MTB-specific antigen that can detect latently infected people from healthy individuals have been obtained (Lalvani, *et al.*, 2001a, Lalvani, *et al.*, 2001b, Lalvani, *et al.*, 2001c). Although the tuberculin skin test is used for the detection of infection due to MTB, it cannot easily discriminate between latently infected and BCG vaccinated individuals (Chaparas, *et al.*, 1970). Furthermore, current treatment regimens for active TB are relatively ineffective against the latent form (Dhillon, *et al.*, 1998). Similarly, BCG vaccine, that has a substantial impact on severe forms of TB in children such as miliary TB or TB meningitis (Rodrigues, *et al.*, 1993) seems neither to prevent latent TB infection nor the reactivation of a latent infection (Dhillon and Mitchison, 1994). Theoretically, it may enhance the latter explaining the negative effect it had in the final assessment of the South Indian trial (Stanford, 1983).

#### 1.3.1.1 Pathogenesis of tuberculosis – Sequence of events

The infectious dose, the number of bacilli per infectious particle and the number of particles inhaled at any one period of exposure are very low in tuberculosis. The droplet nucleus that escapes the defence mechanisms of the respiratory tract of humans is not larger than 5  $\mu\text{m}$  and it is estimated that the number of bacilli present in this droplet nucleus is of the order of 1-10. A low infectious dose was supported further by the examination of X-ray films of a large number of humans (Balasubramanian, *et al.*, 1994b), which revealed that the number of calcified primary lesions is usually one and never more than three.

Following inhalation, virulent microorganisms lodge in the alveoli or terminal air passages of the lung, where they are taken up by the alveolar macrophages. In a

few people, those who have a more effective alveolar macrophage population, this defence mechanism is strong enough to destroy or inhibit the multiplication of the MTB, and the disease is contained within 2-10 weeks (Fig 1.1).

In susceptible individuals, the droplet nucleus is ingested by an alveolar macrophage at the site of implantation, and after a lag period of few days, the bacillus multiplies intracellularly. The macrophage eventually dies and the bacilli are released and ingested by other alveolar and non-activated macrophages, i.e., macrophages transformed from blood monocytes. Once, the host immune system recognises the presence of bacilli it activates a tissue-damaging immune response, which kills the inactivated macrophages. Elimination of bacilli is a very complicated event, which involves T-cells and cytokines. Immunopathology of the granuloma formation and the mechanisms bacilli use to overcome host defence are reviewed in detail in section 1.7. Local destruction of tissues controls the uninhibited intracellular multiplication of bacilli that would otherwise be fatal (Dannenberg, 1991). Thus, by killing the non-activated macrophages, the host eliminates the intracellular environment that is favourable to the growth of the bacilli: this is the first stage of caseation: development of a caseous necrotic centre. As this process continues, a local focus of disease called the 'Ghon focus' forms. From the Ghon focus, bacilli are transported to the lymph nodes at the hilum, by the lymphatics draining the area containing the lesion. Additional foci of disease develop at the hilar lymph node. The Ghon focus, together with the hilar lymphadenopathy is termed the 'primary complex' or 'Ghon complex'. Primary lesions can occur anywhere in the lung (Medlar, 1948). The primary complex can proceed in one of two ways (Dannenberg, 1989) according to the efficacy of the cell-mediated immunity (CMI).

In resistant individuals, who have a competent CMI, activated macrophages accumulate at the caseous focus and prevent the extension of the disease. Being metabolically active, the macrophages consume oxygen diffusing into the lesion so that the central region becomes anoxic. The tubercle bacilli can survive in this calcified nodule, but cannot multiply because of anoxic conditions, reduced pH, and the presence of inhibitory fatty acids (Hemsworth and Kochan, 1978). This is called **inactive tuberculosis infection**. Live bacilli have been isolated from

granulomas or tubercles in the lungs of persons dying of other causes, indicating that the organism can survive in a granulomatous lesion for many years (Opie and Aronson, 1927, Robertson, 1933).

Even though this is the most accepted view of the pathogenesis of TB, it does not explain the presence of MTB DNA in cells other than macrophages, including fibroblasts, endothelial cells, and pneumocyte type II cells of lungs (Hernandez-Pando, *et al.*, 2000). Furthermore, some studies have shown that mycobacteria can be present not only in the calcified lesions but also in histologically normal lung tissues (Opie and Aronson, 1927, Hernandez-Pando, *et al.*, 2000).

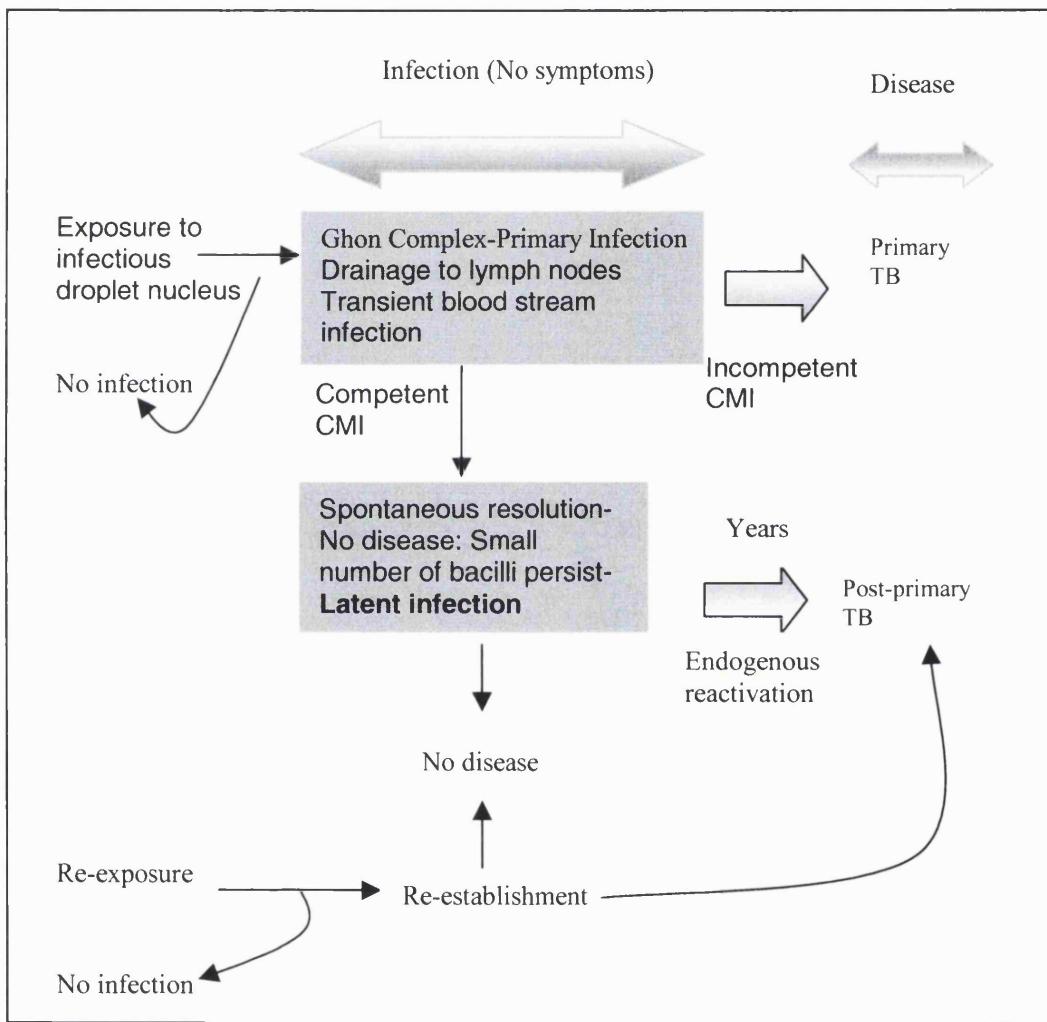
The protective bactericidal mechanism in humans is not yet clear. The general belief is that most healthy people generate a strong immunity to TB, in the process of generating a circulatory memory T-cell population. Mycobacteria resisting the human immune response can exist for years, and any fluctuation or reduction in the state of immunity may set the stage for reactivation of disease. This event is called the **endogenous reactivation** of TB (Smith and Wiegeshaus, 1989).

On the other hand, in a susceptible person who develops only weak CMI, tubercle bacilli released from the edge of the caseous centre are ingested by incompetent macrophages. Therefore, the tissue-damaging immune response, a necrotising delayed type hypersensitivity (DTH), continues in these individuals. These macrophages die and the caseous centres enlarge progressively, and possibly rupture into the pleural cavity resulting in formation of cavitary lesions (tubercles) within the lung, in which the mycobacteria are able to multiply extracellularly. This is the **active contagious form of the disease**, which commonly occurs within 3-8 months after tuberculin conversion according to the skin test. In contrast to the primary lesions, cavitary lesions characteristic of tuberculous disease are always located in the apical region of lungs (Sweany HC, 1931).

### 1.3.1.2 Latent infection - contribution from research

The presence of live MTB after primary infection has been experimentally documented in human lung tissues. Cultivable bacilli from autopsies and surgical specimens of humans who were asymptomatic were demonstrated in the early part of the last century. Opie and Aronson (1927) demonstrated latent TB in lung lesions, fibrocaseous lesions of the pulmonary apex and tissues surrounding caseous or calcified nodules, and in normal looking lung tissue from patients who died from diseases other than TB. They inoculated guinea pigs with suspensions of lung tissue from childhood forms of tuberculosis and reported that the focal pulmonary lesion and associated lymph node yielded live bacilli in 10% of specimens. They also noted tubercle bacilli in lung tissues from apex, base, and tracheobronchial lymph nodes, in which tuberculous lesions were not present. Live bacilli were found in one or other of above-mentioned sites in fifteen of thirty three persons (~50%) examined. A recent study using *in situ* PCR (ISPCR) has also revealed the presence of MTB DNA in lung autopsies from people who died from causes other than TB (Hernandez-Pando, *et al.*, 2000).

The persistence of viable tubercle bacilli in pulmonary lesions representing different stages of development and arrest has been confirmed even after chemotherapy (Wayne, 1956). A significant proportion (at least 20%) of blocked lesions from treated TB patients whose sputum had converted to negative status nine or more months prior to re-sectinal surgery were found to yield small number of colonies of MTB on culture of specimens. These key studies confirmed the persistence of bacilli in lung tissues for a long period, not only in old tuberculous lesions but also in macroscopically and microscopically normal lung tissues in latently infected individuals, as well as in treated TB patients.



**Figure 1.1: Schematic representations of the possible outcome after exposure to communicable TB.**

### **1.3.1.3 Haematogenous dissemination and location of dormant bacilli**

#### **(a) Haematogenous dissemination**

Dissemination of viable bacilli from alveolar and non-activated blood-borne macrophages into the lymph or circulatory system is critical for the establishment of disease. According to some TB specialists, after establishing initial infection, eventually bacilli residing inside the infected phagocytes escape the swollen node to enter the blood stream and to produce a bacteraemia (Stead, 1967, Russell, 2001, Bermudez, *et al.*, 2002). This view is supported further by a study of an animal model (Smith, *et al.*, 1970). These investigators infected guinea pigs through the respiratory route using a very low dose and checked all six lobes of the lung at different stages of the infection for live bacilli. While only lobes with primary infection yielded live bacilli 16 days after infection, they detected live bacilli 22 days after infection in lobes where primary lesions had not been detected. This observation indicates that haematogenous seeding of the initial infection certainly occurred. Furthermore, fresh tubercles, which appeared at the latest study intervals showed no relationship to the location of the primary lesion.

Seeding of bacilli into the bloodstream during initial infection with virulent tubercle bacilli takes place by way of the efferent lymphatic, which drains the lymph node component of the primary complex. The bacilli enter the bloodstream through the thoracic duct. From there, they are taken to other organs, including the brain and this type of haematogenous spread of tubercle bacilli may cause extra-pulmonary TB.

Although most published studies on intracellular infection have focused on the macrophage as the primary host cell, several laboratories have demonstrated that MTB is perfectly capable of entering and replicating in non-professional phagocytes (Bermudez and Goodman, 1996, Menozzi, *et al.*, 1996). MTB expresses a heparin-binding hemagglutinin (HBHA) that is capable of mediating adherence and entry into non-professional phagocytes such as epithelial cells - most notably type II pneumocytes (Pethe, *et al.*, 2000). These authors also noted

that while mutants for HBHA behaved as wild type in growth in liquid culture or inside cells, their invasion into pneumocytes was greatly impaired. Bacterial enumeration in lungs and spleen of mice challenged intratracheally with both wild type and HBHA mutant organisms had revealed that even though colonisation levels in the lung were identical, mutants showed a severe defect in their ability to disseminate and colonise other organs. These data imply a role for HBHA in early invasion through non-professional phagocytes.

Transcytosis assays conducted with pneumocyte monolayers have indicated that MTB is internalised with low efficiency by cells and subsequently excreted at the basolateral surface of the cell (Birkness, *et al.*, 1999). Therefore, it has been suggested that dissemination after initial infection might involve direct binding and entry into the pneumocytes, transcytosis across the alveolar layer and delivery to macrophages migrating into the tissue from blood vessels or possibly into the blood vessels themselves.

However, a recent *in vitro* study on mechanisms of translocation of MTB through epithelial and endothelial cells has revealed that it is not as simple as was thought. Bermudez and colleagues developed a two-layer transwell system to study the ability of MTB H37Rv to cross and disrupt the basal membrane (Bermudez, *et al.*, 2002). They noted that while MTB invaded A549 type II alveolar cells with an efficiency of 2 to 3% of the initial inoculum, it was not efficient in invading endothelial cells, suggesting the need for specific interaction (Pethe, *et al.*, 2000). However, if MTB was passaged in A549 cells or human macrophages, subsequently, endothelial cells took them up with an efficiency of 5 to 6% of the inoculum. These data imply that an invasive phenotype might have emerged when the bacterium was exposed to the intracellular environment. They also noted that infected monocytes crossed the barrier with greater efficiency when A549 cells were infected with MTB than when A549 cells were not infected. Previous studies have demonstrated that A549 cells infected with MTB H37Rv induce the release of chemokines (Lin, *et al.*, 1998). Chemokines create a gradient between the alveolar side and the endothelial side, thereby stimulating monocyte migration. Monocytes infected with MTB trigger the release of tumour necrosis factor alpha (TNF- $\alpha$ ) (Barnes, *et al.*, 1993), which induces an

inflammatory reaction and tissue damage. This could facilitate spread of the bacteria either extracellularly or intracellularly into the blood stream – another possible mechanism of dissemination. However, it should be noted that TNF- $\alpha$  is not toxic in the presence of the Th1 response, unless a degree of Th2 responsiveness is also present.

Therefore, it is clear that MTB uses more than one mechanism to quickly get in to the tissue macrophages and from there disseminate effectively before the host can mount an immune response.

(b) Location of non-replicating bacilli within the lungs and endogenous reactivation

In humans, most of the primary lesion and associated lymph nodes are eventually sterilised; fewer than 10% of examined tuberculous lesions yielded live bacilli (Opie and Aronson, 1927). The restricted location of single cavitary lesions in active TB suggests that complete sterilization of lesions does not occur in the apical region of the lungs. Medlar also suggested that 'certain areas of the pulmonary lobes, upper half of the lobe and infraclavicular regions, are especially subjected to the development of progressive tuberculosis'. He referred to these areas as the 'vulnerable regions' (Medlar, 1948). Hernandez Pando and colleagues also reported the presence of MTB DNA in 29% of macroscopically and microscopically normal lung tissues sampled from the hilar region and apex near to the pleura (Hernandez-Pando, *et al.*, 2000). These observations suggest that reactivation of endogenous TB arises not from any of the arrested primary lesions in the lungs, but rather from the progeny of bacilli from the primary lesions that had been disseminated haematogenously to the apical region of the lung at the time of bacteraemia (Balasubramanian, *et al.*, 1994a).

(c) Cellular location of non-replicating bacilli

It is not known how many alveolar macrophages the bacilli might encounter as they enter the alveolar space. It has been estimated that in an average male there are 28,000 type I pneumocytes, 1400 type II pneumocytes and 50 to 100 alveolar macrophages per alveolus (Bermudez, *et al.*, 1997). This, the bacilli might well interact initially with epithelial cells, lining the alveolar space. *In vitro*

studies on invasion and intracellular replication of MTB have shown that bacilli enter and replicate in lung epithelial cells, and endothelial cells (Bermudez and Goodman, 1996, Mehta, *et al.*, 1996). A study described in this thesis also revealed presence of MTB DNA in non-professional phagocytes (Hernandez-Pando, *et al.*, 2000). Being located in non-professional phagocytic cells may be a devise bacilli use to overcome the host immune defence. Non-professional phagocytes may be the protective sites for MTB to exist unperceived, as these are located away from the bactericidal activity of the macrophages.

(d) Metastatic site of non-replicating bacilli

Location of the latent bacilli is still a mystery. Even though their presence was established in old pulmonary granulomatous lesions or pulmonary lymph nodes, this does not explain the phenomenon of extra-pulmonary TB (TB in the bone, brain, lymph nodes or other tissues). As patients with extra pulmonary TB do not present with lung disease, the origin of reactivation is likely to be from an extra-pulmonary site. Hence, it is clear that TB of all internal organs other than the respiratory tract and alimentary tract, and their associated lymph nodes, must be endogenous and haematogenous in origin. The bacilli must have been carried to these organs from primary lesions, which were disseminated haematogenously at the time the primary complex was formed. Evidence of dissemination during primary infection has been shown by the presence of tubercle in the distal organs of human beings, when the body contained a fresh primary complex (Balasubramanian, *et al.*, 1994b). Therefore, there is every reason to believe that, latency is maintained in widely disseminated sites rather than confined to the lung. To date, haematogenous seeding of MTB and development of latent infection in the distant organs has not been documented. In this study, efforts have been made to address this unsolved puzzle.

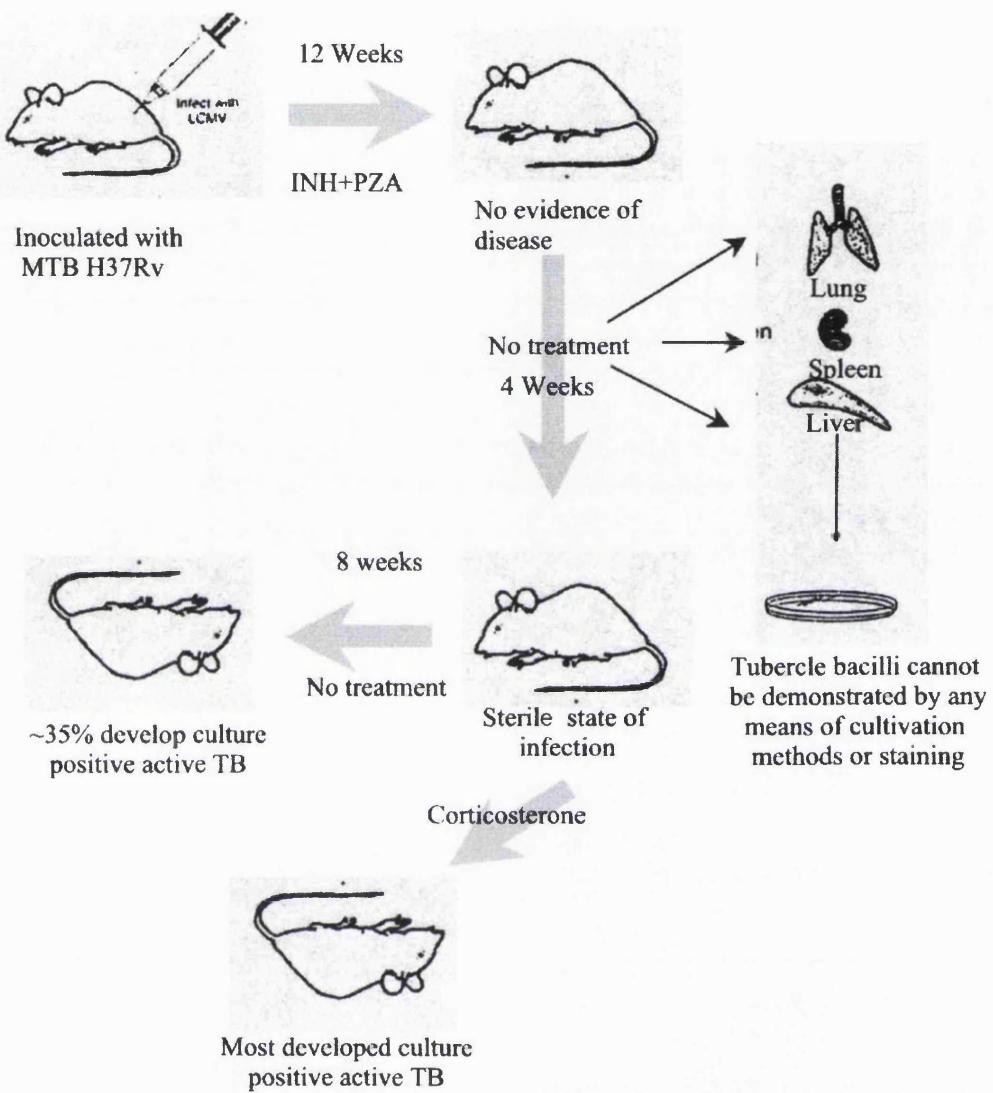
### **1.3.2 Animal and bacteriological models of latent tuberculosis infection**

Many animal and bacteriological models of MTB latency have been developed and used extensively in attempts to characterise latency.

### 1.3.2.1 Experimental animal infections

Animal models have been used to demonstrate both immunologically-induced latency and post-chemotherapeutic latency, with the associated late reactivation of disease. McCune et al (1966 a, b) first studied the post-chemotherapeutic latency of tubercle bacilli, in their work described as the "Cornell Model" after the academic institution where the work was done. In this study, they infected mice intravenously with a dose of  $10^5$  virulent tubercle bacilli (H37Rv) and the resultant infection was treated for 12 weeks with the anti-mycobacterial drugs isoniazid (INH) and pyrazinamide (PZA) beginning 20 minutes after infection (McCune, *et al.*, 1966a, McCune, *et al.*, 1966b). There is no evidence of disease after 12 weeks of treatment (fig 1.2 (b)). Untreated mice died due to progressive disease 20 weeks after initial infection. During the progression of infection, lung and spleen cultures of treated mice were done at regular intervals. After 12 weeks of treatment, it was shown, on culture and upon injection of mouse tissue into additional mice or guinea pigs, that the organs became apparently completely sterile or the number of organisms was reduced to an undetectable level 'sterile state' (Fig 1.2 (c)). In addition, smears from the sterile state only very rarely revealed acid-fast forms, after examination of thousands of fields under a high power microscope. However, 12 weeks after cessation of antibiotics, approximately one-third of the mice developed full-blown active TB, with nearly two-thirds displaying disease after 24 weeks (McCune, *et al.*, 1956a).

Using the same model, McCune et al. have later shown that administration of cortisone, a broad immune suppressant, some times after withdrawing chemotherapy (fig 1.2 (c)) reduced the time required for 50% of the mice to revert to a culture-positive state from 7 to 2.5 months (McCune, *et al.*, 1966a, McCune, *et al.*, 1966b) (Fig 2 (e)).



**Figure 1.2: Schematic diagram of the Cornell model of dormant tuberculosis (TB)**

(a) Out bred mice are inoculated with  $\sim 10^5$  colony-forming units (CFU) of the H37Rv strain of *M. tuberculosis* (MTB). (b) After treatment with the antimicrobial drugs isoniazid (INH) and pyrazinamide (PZA) for a period of 12 weeks, there is no evidence of disease. (c) Four weeks after withdrawing INH and PZA, the mice appear to be well, and the tuberculosis infection cannot be demonstrated by any known cultivation methods. This is termed 'sterile' state of latent TB. (d) Eight weeks later, however,  $\sim 35\%$  of the mice develop culture-positive active TB. Adapted with modification from Parrish et al (Parrish, et al., 1998).

Using molecular technology Mitchison and colleagues further confirmed the persistence of tubercle bacilli after effective treatment of MTB in the so-called 'sterile state mice'. They reproduced the sterile state in mice using the Cornell protocol and tested the sterile state tissues for MTB DNA by the polymerase chain reaction (PCR) and dot blot hybridisation (de Wit, *et al.*, 1995). A significant result from this work was the detection of  $\sim 10^5$  genomic equivalent mycobacterial DNA/ml of tissue. Since, microscopic examination of sterile state tissues from the Cornell model sometimes fails to reveal acid-fast bacilli and did not yield growth by any means of culture, they concluded that persistence of significant quantities of MTB DNA may be from dead disintegrated bacilli, or from dormant bacilli, that may be in a non-acid-fast form. However, recently it has been proved that the bacilli in the sterile state mice are viable but not cultivatable (Pai, *et al.*, 2000). Whether these bacilli are uncultivable is still a subject of debate. It is uncertain whether the bacilli during latent infection require a longer incubation period *in vitro* to be detectable, because it was reported that tubercle bacilli from pulmonary lesions resected from humans emerged late in liquid cultures (Hobby, *et al.*, 1954). Hobby and colleagues employed special processing of resected samples, and neutralised inhibitors found in the necrotic material, which enabled them to demonstrate viable bacilli in approximately 78% of the closed or healed lesions.

The Cornell model is an attractive model of human latent tuberculosis infection (LTBI) because of the low bacterial burden, at undetectable level, in the mice, which is not cultivatable using any known culture method (fig 1.3(b)). In an autopsy series in which tissues from treated and untreated patients were examined, acid-fast bacilli were visible but not cultivatable (Medlar, 1948). The Opie and Aronson study also revealed that while examination of pulmonary lesions of latently infected people rarely revealed acid-fast bacilli, homogenised pulmonary lesions that were injected into guinea pigs were capable of causing infection (Opie and Aronson, 1927). Thus, undetectable bacteria during the 'sterile state'/chronic infection in the Cornell model equates to latent infection in humans. However, the introduction of antibiotics to reduce the bacterial numbers after initial infection does not mimic the situation in natural human LTBI. Furthermore, in many earlier studies with this model antibiotics were

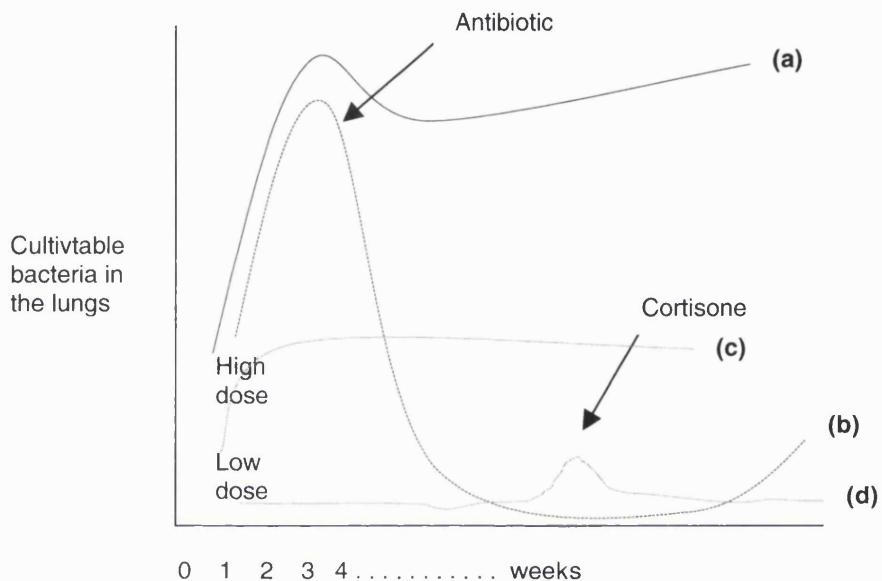
administered immediately after infection and this could have even inhibited the first cycle of replication of the bacilli after initial infection. Therefore, it is not clear how many bacteria would have survived the treatment to cause the infection.

Variations of the Cornell model, using different doses of bacilli to infect the mouse given either via the intravenous or aerosol routes; different initiation time of drug therapy; and drug therapy for different periods of time with different drug regimens; have been extensively used to study persistence and reactivation of mycobacteria in the host. The above-mentioned animal models are also being used to develop a vaccine for latent infection (Brooks, *et al.*, 1999, Lowrie, *et al.*, 1999, Scanga, *et al.*, 1999). These studies have focused more on the ability of the mycobacteria to persist or replicate following antibiotic regimens and less on the role of the host immunity in maintaining a latent infection. Furthermore, antibiotic treatment may have a direct effect on the development of a protective immune response.

Another animal model widely used to study human LTBI was established by infecting relatively resistant mouse strains (such as C57BL/6) via aerosol or via the intravenous route using an appropriate dose (Orme, 1988, Flynn, *et al.*, 1998). In these mice, the immune system controls the growth of the bacteria and bacterial numbers reach a plateau (fig 1.3 (c)) a few weeks after initial infection. Even though the immune system fails to reduce the bacterial numbers in the lung or spleen after this point, mice do not show clinical signs of disease. In fact, a chronic infection is maintained for many months. It is an attractive model as it truly represents an equilibrium between host and bacillus and because bacterial numbers are controlled by the immune response, which is also true for human LTBI. The shortcoming of this model is the high bacterial numbers in the lung, which do not reflect the situation in humans.

Recently an immunologically induced model, avoiding the use of anti-tuberculosis drugs, was developed using a low infection dose ( $4 \times 10^3$ ) via intratracheal injection (Arriaga, *et al.*, 2002) and microbiological and histopathological changes were studied. In intatracheally-infected mice, the bacterial load in the lungs was kept relatively low by the immune system and the bacterial number was stable

270 days post-infection (fig 1.3 (d)). In addition, very few histological changes were noted: scattered areas of activated macrophages and lymphocytes without neutrophils in alveolar lumens were observed in the lung of latently infected mice. Interestingly, *in situ* PCR revealed MTB DNA not only in areas of lung where there were histological changes, but also in histologically normal areas. This observation well reflects the latent infection in the human (Hernandez-Pando, *et al.*, 2000).



**Figure 1.3: Tuberculosis in the mouse.**

Mice infected with a high dose show an initial rise in bacterial numbers in the lungs (acute infection) followed by clinical symptoms and an increase in bacterial burden (a), nevertheless if treated with antibiotics the bacterial burden decreases and reaches an undetectable level. However, 12-24 weeks after withdrawal of antibiotics clinical symptoms reappear and bacterial burden increase (b). In relatively resistant mice, infected with a low dose, the bacterial burden grows to about  $10^5$  bacilli/total lung, and gradually moves into a chronic infection state because of acquired immunity/chemotherapy and does not change much until the animal becomes old. (c). The plateau phase in c) or more probably in d) may be equivalent to the latent infection phase in human tuberculosis. In mice, infected with a very low dose (4000 bacilli) there is little change in the bacterial burden after infection (d). Introduction of cortisone to model (d) after the establishment of histological changes may more accurately model reactivation of latent infection in humans.

This is an attractive model as the bacterial burden is controlled by the immune system and histological changes are less. Nevertheless, the bacterial burden was multibacillary (the bacteria are easy to isolate and culture in large numbers), compared to human LTBI, which is paucibacillary (bacteria are difficult if not impossible to find by staining or culturing).

Despite the difficulty in modelling human latency in experimental animals, the understanding of both host and microbial factors that contribute to the establishment and maintenance of a plateau bacterial burden during infection has progressed and the information gathered is pertinent to human latent TB.

### **1.3.2.2 *In vitro* model of anaerobic tubercle bacilli during latent infection**

The mycobacteria in lung lesions during latent infection are subjected to many adverse conditions. Lawrence Wayne conducted pioneering studies of the bacterial persistence of MTB and developed an *in vitro* model (Wayne, 1976). The rationale behind this experiment is that lung granuloma is the sanctuary for non-replicating MTB and the gradual oxygen depletion during the course of granuloma formation may trigger bacterial adaptation to the latent state.

In this model, cessation of the rapid growth then death and autolysis of most of the bacilli occurred when the aeration of a vigorously agitated logarithmic phase culture of MTB in glycerol-rich medium was interrupted, followed by rapid and severe O<sub>2</sub> depletion. On the other hand, replication of bacilli stopped when they settled slowly through an O<sub>2</sub> gradient and this may be the case with MTB in granuloma, but viability did not appear to be impaired for at least several weeks. The adapted sediment, on re-suspension and aeration, exhibited an immediate acceleration of RNA synthesis (Wayne, 1977). Although several biochemical events were found to be associated with the shiftdown the timing and sequence of these events could not be established because of the heterogeneity of the transitional settling cell.

Thus, to study the sequential events that characterise the process of shiftdown in the heterogeneous population of settling bacilli, Wayne developed a model system that is based on controlled agitation of sealed liquid cultures exposed to

limited head space volume: gradual depletion of oxygen (Wayne and Hayes, 1996). In this study when dissolved O<sub>2</sub> level approached 1%, organisms were found to shift from rapid to slow replication and the stage was designated as the non-replicating persistence stage 1(NRP 1). Bacilli adapt to microaerophilic conditions by synthesising an antigen designated URB, now identified as the *acr* gene (Yuan, *et al.*, 1996), coding for alpha crystalline, a small heat shock protein and by producing two enzymes, isocitrate lyase; (Wayne and Lin, 1982) and nitrate reductase (Wayne and Hayes, 1998). Isocitrate lyase, the first enzyme in the alternate glyoxylate pathway, enables them to bypass the CO<sub>2</sub>-generating steps of the tricarboxylic acid cycle (TCA) (McKinney, *et al.*, 2000) and nitrate reductase serves mainly a respiratory function to generate energy in the absence of oxygen (Wayne and Hayes, 1998).

Synthesis of DNA and, to a large extent, of RNA, stops abruptly during NRP1 (Wayne and Hayes, 1996). MTB are resistant to the bactericidal action of drugs that target enzymes responsible for the synthesis of bacterial components: e.g. ciprofloxacin which inhibits the A subunit of gyrase required for DNA synthesis, and isoniazid, which inhibits cell wall synthesis.

When the dissolved O<sub>2</sub> content of the culture fall further, to 0.06% saturation or below, the bacilli shift down abruptly to an anaerobic stage, designated non-replicating stage 2 (NRP 2), and exhibited a 10-fold increase in synthesis of glycine dehydrogenase. Glycine dehydrogenase converts glyoxylate to glycine and thereby diverts glyoxylate out of the TCA cycle. Diversion of some of the glyoxylate into the reductive amination pathway can serve to regenerate NAD from NADH, which is required to meet the needs of compensating dehydrogenase reactions under oxygen depletion. Regeneration of NAD may provide energy for further, as yet unidentified, steps in the shiftdown of MTB.

Prevalence of the anaerobic condition is further supported by the susceptibility of bacilli in the dormant state to metronidazole, a drug that is known to act on anaerobic bacteria (Wayne and Sramek, 1994). However, mice with chronic disease do not respond to metronidazole therapy (Brooks, *et al.*, 1999). It should be noted here that the metronidazole only kills bacilli under strictly anaerobic

conditions, and does not work under the initial stage of NRP that presumably corresponds to the state of the bacilli *in vivo*.

The knowledge gained through the above-described models is relevant to those bacilli that survive in the granuloma during latent infection. This does not explain the observations made by Opie and Aronson and the thesis author, of the presence of bacilli in well-oxygenated tissues during latent infection.

Non-replicating bacilli in the calcified lung lesions are not only  $O_2$ -starved, their accessibility to other nutrients is also limited (Nyka, 1974). Nyka starved MTB cells for 6 months, either in plain agar or just in distilled water, and showed that they differed in their morphology and staining properties from those growing in cultures. *In vitro*-starved cells were small spherical cells rather than rods and were chromophobic, i.e. they were not stained with conventional stains and were not acid fast (Nyka, 1967). When they were added to a nutrient-rich liquid medium, even after 2 years of starvation, they regained their acid fastness and started growing (Nyka, 1974). Therefore, it is possible that this type of cell could be obtained from latent lung lesions and this may be the case in the Cornell model sterile state, where no acid-fast bacilli could be found.

In the latter experiment, limiting nutrients that kept MTB in the stationary phase during the latent phase of the disease were not studied. However, in 1999, Williams and colleagues used the stationary-phase survival of *M. smegmatis* as a model for the mycobacterial non-replicating state to study the physiological changes that occur during starvation of *M. smegmatis* for carbon, nitrogen, or phosphorus (Marjan, *et al.*, 1999). They have reported reductive cell division on carbon limitation, and resistance to acid and osmotic stress upon entry into the stationary phase from the exponential phase. MTB may also use the same adaptive response to overcome the hostile environment in the granuloma.

## 1.4 Genes associated with mycobacterial persistence

In recent years expression of several genes unique to the stationary phase/non-replicating stage has been identified which can be grouped into three basic categories: respiratory enzymes, stress related products and metabolic enzymes; and, proteins involved in fatty acid metabolism (Table 1.1).

Rv number	Name	Putative function
1736c	<i>narX</i>	'Fused' nitrate reductase, anaerobic respiration
1161-1163	<i>narGH</i> <i>IJ</i>	Nitrate reductase complex, anaerobic respiration
1737c	<i>nark</i> <i>2</i>	Nitrite-extrusion protein
2031c	<i>hspX</i>	16-kDa homologue, alpha crystallin
3286c	<i>sigF</i>	Alternate RNA polymerase sigma factor
0353,0350	<i>hspR, hsp70</i>	HspR repressor, heat shock protein
0467	<i>icl</i>	Isocitrate lyase, glyoxylate cycle
0470	<i>pcaA(umaA2)</i>	Cyclopropane mycolic acid synthase
1908c	<i>katG</i>	Catalase-peroxidase

**Table 1.1: Genes with implicated function in persistence of *Mycobacterium tuberculosis* complex**

### 1.4.1 Respiratory enzymes

According to Wayne's *in vitro* model, bacilli undergo a metabolic shift down under hypoxic conditions and adapt their metabolism to anaerobiosis by switching to nitrate respiration (Wayne and Hayes, 1998) and reductive amination of glyoxylate (Wayne and Lin, 1982).

Little was known about the control and function of nitrate reductase, an enzyme allowing nitrate respiration in *Escherichia coli*, of MTB until the complete genomic

sequence was available. Earlier it was thought that function of nitrate reductase in MTB is associated with assimilation of nitrogen. However, in *E.coli* and other anaerobic bacteria it serves as a respiratory-linked enzyme in which nitrate is used as an alternative electron acceptor to oxygen. The genome of MTB H37Rv contains two loci that have homology to prokaryotic respiratory nitrate reductase.

One of them is *narGHJI*. Cloning of this gene conferred anaerobic nitrate reductase activity to *Mycobacterium smegmatis* (Weber, *et al.*, 2000) suggesting its function in nitrate respiration. Furthermore, insertional mutants of *narGHJI* in a previously nitrate reducing strain of *M. bovis* BCG lost nitrate reductase activity. When this mutant strain was used to infect severe combined immunodeficient mice, they showed smaller granulomas with a lower bacterial burden than those infected with wild type, suggesting that this locus is important for pathogenesis (Weber, *et al.*, 2000). Another study also showed the importance of the *narGHJI* locus for the pathogenesis of *M. bovis* BCG. These investigators observed that *M. bovis* BCG lacking *narGHJI*, failed to survive in the lungs, liver and kidneys of immunocompetent (BALB/C) mice (Fritz, *et al.*, 2002). They also noted that the plateau phase and growth of BCG in the spleens of either mouse strain appeared largely unaffected by lack of anaerobic nitrate reductase, indicating that the role of the enzyme in pathogenesis is tissue-specific.

Another gene found to be up-regulated under hypoxic conditions was *narX*, encoding putative 'fused nitrate reductase' (Hutter and Dick, 1999). This gene shows homology to various subunits of the multimeric nitrate reductase *narGHJI* and has several putative transmembrane domains at the C-terminus. This indicates that it could be a transmembrane protein and might be associated with the electron transport chain, in other words it might function as a respiratory nitrate reductase.

These observations suggest that nitrate respiration may be a mechanism by which MTB survives under hypoxic conditions, by which it can reduce the elevated levels of nitrate that can occur as an NO degradation product in O<sub>2</sub> depleted areas of inflammatory or necrotic tissue. Reactive nitrogen

intermediates play an important role in the control of experimental TB (Flynn, *et al.*, 1998).

Nitrite, a reduced form of nitrate is not reduced by MTB in any stage of adaptation (Wayne and Hayes, 1998). This is an unstable form compared to nitrate and may be secreted into the environment by *narK2*, a putative nitrite-extrusion protein that has been found to be up-regulated under hypoxic conditions (Hutter and Dick, 2000). Expression of nitrate respiration genes would provide MTB with a distinct survival advantage under the hostile environment they have to face in the granuloma.

#### **1.4.2 The stress responses and other metabolic products**

Among the changes that accompany the shift of aerobic cultures to the hypoxic NRP state is induction of the alpha crystallin homologue protein, which has not been detected outside the TB complex (*M. tuberculosis*, *M. africanum* *M. microti* *M. cannetti*, *M. bovis* and *M. bovis* BCG) (Yuan, *et al.*, 1996). The alpha crystallin homologue, also referred to as URB-1 antigen; 14K antigen; the small heat shock protein sHsp 16; Hsp 16.3; 16kDa and *acr* or *hspX* gene, is produced when the bacilli encounter hypoxic conditions (Wayne, 1994, Yuan, *et al.*, 1996) or on exposure to nitric oxide (Garbe, *et al.*, 1999).

It has been suggested that the 16kDa protein plays a protective role as a chaperone based on its ability to prevent thermal denaturation of alcohol dehydrogenase and aggregation of citrate synthase *in vitro* (Garbe, *et al.*, 1999). Over-expression of *acr* in MTB resulted in slower growth and a slower decline in viability after growth stopped on entry into stationary phase (Yuan, *et al.*, 1996). Yuan and colleagues also demonstrated synthesis of 16kDa at a low level in logarithmic phase cultures, and a marked increase in synthesis during the transition from log phase to stationary phase.

One of the protective mechanisms of 16kDa protein could be correlated with thickening of the cell wall during late stationary phase. When subjected to anaerobic stress, MTB and *M. bovis* BCG induce a massive up-regulation of the production of this protein, which is associated with a thickened cell wall

(Cunningham and Spreadbury, 1998). The 16kDa protein was strongly associated with the cell envelope, fibrous peptidoglycan-like structures, and intracellular and peripheral clusters. A thickened cell wall may play a role in stabilising cell structures during long-term survival, thus helping the bacilli survive the low oxygen tension in granulomas. It would be interesting to know the acid-fast nature of those bacilli with a thickened cell wall.

Recently, regulators of 16kDa expression have been documented. Using whole genome microarray, 100 genes have been identified whose expression is rapidly induced in response to hypoxic conditions. Among the induced genes is an apparent operon that includes the putative two-component response regulator pair Rv3133c/Rv3132c. When the expression of this operon is disrupted by targeted disruption of the upstream gene, the hypoxic regulation of *acr* is eliminated. These results suggest a possible role for Rv3132c/3133c/3134c in mycobacterial latency (Sherman, *et al.*, 2001).

One recent study has revealed the essential role of 16kDa for the survival of bacilli within the macrophages *in vitro*. *acr* expression was found to be induced during the course of *in vitro* infection of macrophages. The mutant strain for this gene was shown to be equivalent to wild-type H37Rv in *in vitro* growth rate and infectivity but was significantly impaired for growth in both mouse bone marrow derived macrophages and THP-1 cells (Yuan, *et al.*, 1998). In addition to its proposed role in maintenance of long-term viability during latent, asymptomatic infections, these results establish a role for the Acr protein in replication during initial MTB infection. The immunodominance of the 16kDa antigen in both the murine and human systems has been documented (Jackett, *et al.*, 1988, Lee, *et al.*, 1992, Vordermeier, *et al.*, 1993, Friscia, *et al.*, 1995, Wilkinson, *et al.*, 1998). Antibody to 16kDa is found in a high percentage of TB patients, suggesting hypoxic shift down of tubercle bacilli to the NRP state *in vivo*. In this thesis, antibody response to 16kDa antigen was investigated in people presumed to be latently infected to evaluate the sero diagnostic value of this antigen for diagnosis of latent infection.

Reactive nitric oxide intermediates (RNI) stress also induces 16kDa protein expression. Importance of RNI in the control of MTB in the murine systems has been well documented by the demonstration of reactivation of MTB growth in mice that do not produce nitric oxide (Flynn, *et al.*, 1998). There are still controversial views regarding the role of RNI in human TB. Nevertheless, nitric oxide synthase has been recently detected in alveolar macrophages fixed immediately upon isolation from TB patients. Exposure of MTB to a panel of five structurally diverse NO donors collectively known as Nox, resulted in induction of 16kDa protein (Garbe, *et al.*, 1999). Furthermore, mutation in the *acr* gene impaired survival of MTB in macrophages. Coincidental expression of 16kDa and the respiratory type of nitrate reductase, suggests a role for 16kDa in a protective mechanism against RNI.

Another group of genes that are found to be associated with adaptation of the bacilli to various environmental conditions are alternative sigma factors. The *sigF* gene is induced under a variety of stress conditions, such as cold/heat shock; nutrient depletion; exposure to antibiotics, in particular to ethambutol; rifampin; streptomycin; and cycloserine; entry into macrophages, and on entry into stationary phase *in vitro* (DeMaio, *et al.*, 1996, DeMaio, *et al.*, 1997, Michele, *et al.*, 1999, Chen, *et al.*, 2000).

*sigF* gene of MTB shares significant homology with the *sigF* of *Streptomyces coelicolor* and *Bacillus subtilis*, which is a sporulation-specific sigma factor and to *sigB* of *Staphylococcus aureus*, a sigma factor that participates in methicillin resistance (Chen, *et al.*, 2000). It is not clear yet, whether *sigF* of MTB also has a similar function. However, it was found to be involved in the expression of *hspX* gene, coding 16kDa protein, (Manabe, *et al.*, 1999), which in turn contributes to the thickening of cell wall of MTB and *M. bovis* BCG during the late stationary phase (Cunningham and Spreadbury, 1998). Chen and colleagues also noted that the 16kDa protein was completely absent in *sigF* knockout mutant stationary phase cultures suggesting that alpha crystallin expression requires *sigF* (Chen, *et al.*, 2000). *sigF* knockout mutants did not show any difference either in short term intracellular growth or susceptibility to lymphocyte-mediated inhibition of intracellular growth compared to the wild type, suggesting that *sigF* is not

necessary for intracellular survival of MTB (Chen, *et al.*, 2000). This latter observation is surprising as it was shown that the 16kDa protein is important for survival in a human macrophage-like cell line and in primary mouse macrophages (Yuan, *et al.*, 1998). Nevertheless, other studies have shown that *sigF* may not be the sole regulator of *hspX* expression and many of the conditions that up-regulate *sigF* expression has no effect on the expression of *hspX* (Yuan, *et al.*, 1996). *sigF* knockout mutants were not as virulent as the wild type of bacilli, because death of BALB/c mice infected by the mutant was significantly delayed (Chen, *et al.*, 2000).

Expression of all the above-mentioned genes favours the bacilli over the host, and enables them to survive during the chronic phase of the tuberculosis infection. However, it appears that not all the proteins of MTB that are expressed/induced under stress favour the bacilli. Over-expression of Hsp70, a stress response-related heat shock reduced the survival of an Hsp70 repressor mutant ( $\Delta$ hspR) in a murine model (Stewart, *et al.*, 2001). Even though the mutant was as virulent as the wild type during the initial stage of the disease, it showed impaired survival during the subsequent plateau phase of the infection. This may be due to the early recognition of secreted proteins by the host immune system.

#### **1.4.3 Proteins involved in fatty acid metabolism**

A recent study done by McKinnney and colleagues has shown that changes in the microenvironment that occur during the course of granuloma formation requires the bacilli to alter their diet from carbohydrate to lipid (McKinney, *et al.*, 2000). They tested the ability of the wild type and the mutant for the *icl* gene, coding for the enzyme isocitrate lyase, to infect mice. In relatively resistant mice, infected with wild type MTB, the initial increase (acute phase) of the bacterial burden in the lung is followed, from about two weeks after infection, by a plateau in bacterial numbers. The mutated isocitrate lyase gene had no effect on the bacterial burden in mice during the acute phase of infection, but from two weeks onwards, the numbers decreased rather than maintaining a plateau, indicating the need for the isocitrate lyase gene for bacterial survival during late-stage infection. The abundance of genes (>150) encoding enzymes involved in fatty

acid degradation support the suggestion that MTB uses host lipids while growing *in vivo* (Cole, 1998). Fatty acids can supply the cells with precursors for carbohydrate synthesis through the glyoxylate pathway (discussed in detail under section 1.6).

In addition to the genes involved in fatty acid catabolism, the genes involved in fatty acid anabolism are also required for prolonged survival *in vivo*. Formation of serpentine cords is a morphological characteristic of virulent MTB. Cyclopropane in the cell wall has been shown to be involved with the formation of cords. The *pcaA* gene coding for cyclopropane synthase is required for synthesis of the cyclopropane ring of mycolic acid. A bacillus mutant for *pcaA* failed to survive in mice. *M. bovis* BCG mutants for *pcaA* have grown as do the wild type in mice, but are progressively eliminated from the animal after the onset of immunity at 3 weeks (Glickman, *et al.*, 2000). Inhibition of cyclopropanation of cell wall mycolic acid can result in change in lipid fluidity and cell wall permeability, and therefore resistance to reactive oxygen and nitrogen intermediates. However, cord factor is also known to induce granulomatous responses in animals and could be an important immune modulator at the infection site. At present, it is unclear if the interesting phenotypes of these mutants result in a bacterium that is attenuated for survival, or a bacterium that is attenuated in its ability to modulate the host response to infection.

Another gene reported to be involved in persistence is PE/PE-PGRS (Ramakrishnan, *et al.*, 2000). These investigators observed expression of a gene homologous to the MTB repetitive PE-PGRS family, in *Mycobacterium marinum*, which is a cause of TB in fish and amphibians and reptiles, when they reside in host granuloma or macrophages. They also noted that mutation in these genes decreased the capacity of the bacilli to survive in the granuloma.

#### 1.4.4 Other genes associated with persistence

Catalase peroxidase, a bifunctional enzyme encoded by the *katG* gene protects tubercle bacilli from organic peroxides including superoxide, hydroxyl radical, and hydrogen peroxide within activated macrophages (Sherman, *et al.*, 1996). Middlebrook and colleagues showed that loss of catalase activity resulted in loss of virulence for guinea pigs and resistance to isoniazid, a drug used in the usual drug regimen for tuberculosis. A recent study confirmed the role of *katG* in the growth and persistence of MTB in both the murine and guinea pig models of human tuberculosis. Transformation of plasmid constructs containing the *katG* gene of MTB into an avirulent *katG*-deleted strain restored its capacity for long-term survival in mouse and guinea pig spleens (Li, *et al.*, 1998), indicating the importance of *katG* for the survival of MTB during the chronic phase of infection.

Another gene that may be involved in controlling growth and survival of MTB *in vivo* is that encoding a resuscitation promoting factor (Rpf). This is a secreted protein, and active at picomolar concentrations believed to act at the cell surface (Biketov, *et al.*, 2000). Five genes encoding a Rpf-like protein were identified in the MTB genome. It is believed that the secreted (Rv0867c, Rv1884c, Rv2389c and Rv2450c) or membrane-anchored (Rv1009) Rpf-like proteins of MTB are surface-located (Cole, 1998). Rpf is an example of a family of autocrine growth factors found throughout the high GC Gram-positive bacteria including MTB (Biketov, *et al.*, 2000). It was purified to homogeneity from *Micrococcus luteus* and was required by its producer to exit from dormancy, defined as an organism in a state of low metabolic activity and unable to divide or to form a colony on an agar plate without a preceding resuscitation (Mukamolova, *et al.*, 1998).

Biketov and colleagues analysed the cultivability of MTB cells isolated from murine macrophages (Biketov, *et al.*, 2000). They noted several magnitudes of difference between the total count and viable counts. This suggests that a substantial proportion of the bacteria obtained from macrophages were not cultivable. Nevertheless, when viable counts of bacilli derived from macrophages were measured using the most probable number (MPN) assay (allows numerical number of viable bacteria by their cultivation in liquid medium at high dilution) the

addition of Rpf in the liquid medium at picomolar concentrations caused a 10-100 fold increase in the viable count. These data imply that some of the mycobacterial cells obtained from macrophages may be in a non-replicating physiological state *in vivo* and Rpf is required to exit from this state.

### **1.5 Morphology of non-replicating tubercle bacilli during latent infection**

A typical tubercle bacillus is 1 to 4  $\mu\text{m}$  long and 0.3 to 0.6  $\mu\text{m}$  in diameter, acid-fast, and non-sporing with a simple life cycle of binary fission. However, aberrant non-acid-fast forms have been reported throughout the history of mycobacteriology, possibly involving more elaborate life cycles and production of minute granular forms, sometimes termed Much's granules or cell wall-free protoplasmic forms (Minchin, 1927, Stanford, 1987).

It is apparent in any infected tissue that the tubercle bacillus grows minimally as an acid-fast rod, showing binary fission. The predominant growth consists of pleomorphic structures and mycobacterial filaments, which divide simultaneously into 10 to 15 bacilli or develop a variety of other forms (Xalabarder, 1958, Xalabarder, 1970). These observations raise a question concerning the morphology of the bacilli. Cell wall defective (CWD) forms of MTB in the blood of TB patients have been reported and, many laboratories find that seeking such forms in sputum greatly increases the sensitivity of TB diagnosis (Gamzaeva, 1989).

Interestingly, the presence of CWD of tubercle bacilli in the sputum of 'cured' cases increased their chance of relapse (Dorozhkova, *et al.*, 1995). Another study has also disclosed that tuberculosis patients who discharge CWD tubercle bacilli are especially contagious (Khomenko and Muratov, 1993). These observations suggest that the bacilli attain an aberrant morphology, possibly a CWD form, under stress, enabling survival. This view is further supported by the presence of viable CWD (filterable forms) of MTB in the lungs of individuals who spontaneously recover from tuberculosis (Khomenko, 1987). Furthermore, there is a body of evidence for the presence of filterable forms in pleural fluid, ascitic

fluid, spinal fluid in meningitis, urine, and in breast milk of TB patients. Inability to demonstrate the presence of bacilli in the 'sterile' state of the Cornell model either by acid-fast staining or by any mean of culture methods may also be an evidence for this view.

MTB obtained from macrophages taken from infected mice showed a range of morphological types including rods, ovoid forms, and coccoid forms (Biketov, *et al.*, 2000). When the bacilli were grown in macrophages, during the first passage, normal rod-shaped cells were converted into ovoid forms (diameter  $1.2\pm0.4$   $\mu\text{m}$ ) and short rods (length  $1.2\pm0.2$   $\mu\text{m}$ ; diameter  $0.6\pm0.2$   $\mu\text{m}$ ). After the second passage, about 90% of the cells were smaller coccoid forms obtained by filtration through a  $0.45$   $\mu\text{m}$  filter. Significant alteration to the surface of bacilli was also observed suggesting cells grown *in vivo* change their morphology on exposure to the intracellular macrophage environment.

TB specialists are very sceptical about the idea of sporulation in MTB allowing it to persist in a hostile environment. MTB may not generate spores, but there may be a connection between the regulatory factors, and alternative sigma factors as seen in spore-forming bacteria (DeMaio, *et al.*, 1997). These regulatory factors might have a role in the adaptation of the cell wall that could favour persistence. One such adaptation could be the 16-kDa protein produced by MTB under stationary conditions. It is possible that this protein forms an additional coat of some sort around the bacterium, which could provide extra protection against host defence mechanisms and drugs (Cunningham and Spreadbury, 1998).

To date, however, there is no evidence to support the suggestion that MTB, persisting during apparently adequate chemotherapy or surviving in the interval between infection and reactivation of disease are non acid-fast and variant. There is no documentation on visualisation of persisters while they are in their natural environment.

## 1.6 Physiology of tubercle bacilli during latent tuberculosis infection

*M. tuberculosis* is an aerobe and uses glycerol and asparagine as its main carbon and nitrogen sources. It generates energy through linking of respiration to oxidative phosphorylation. However, MTB in its natural habitat is exposed to many adverse conditions that require it to adapt by modifying its physiological characteristics.

The physiological state in which the bacilli exist during the latent infection in the granuloma and in the well-oxygenated tissue is still not known: whether they are in a metabolically active state of stationary or usually slow growth or whether there are spore-like metabolically inactive forms awaiting a signal to resume division, are subject of further investigation.

McKinney and others recently reported the importance of the isocitrate lyase enzyme for bacterial survival during latent infection by generating an MTB strain with a mutation in the isocitrate lyase gene (McKinney, *et al.*, 2000). Isocitrate lyase and malate synthase together form the 'glyoxylate shunt', which bypasses the CO<sub>2</sub>-generating steps of TCA cycle - the metabolic pathway by which acetate is oxidised to generate ATP (Fig 1.4). The net result of the glyoxylate pathway is the consumption of two molecules of acetyl-CoA to generate one molecule of succinate and one molecule of glyoxylate. Lipids are a source of acetyl-CoA through degeneration of fatty acids via the  $\beta$ -oxidation cycle; succinate is a precursor for the synthesis of sugars. Consequently, the glyoxylate bypass allows MTB to utilise fatty acids and to synthesis carbohydrates as well as supply intermediates to support the TCA cycle.

This observation further supports the view that a change of environment that occurs during granuloma formation requires the bacteria to alter their diet from carbohydrate to lipid (C<sub>2</sub> Carbon sources) as the external supply of carbohydrate is stopped. Lipid-rich host-cell debris evolved during granuloma formation and possibly the mycolic acid of MTB cell wall may act as a source of acetyl CoA to generate succinate-precursor for the synthesis of glucose through the 'glyoxylate shunt' pathway.

However, if the MTB lurks in a well-oxygenated tissue during latent infection then it will not encounter either food shortage or oxygen deficiency. Therefore, it is not yet clear in which ways they adapt themselves to such an environment and remain in a non-replicating state.

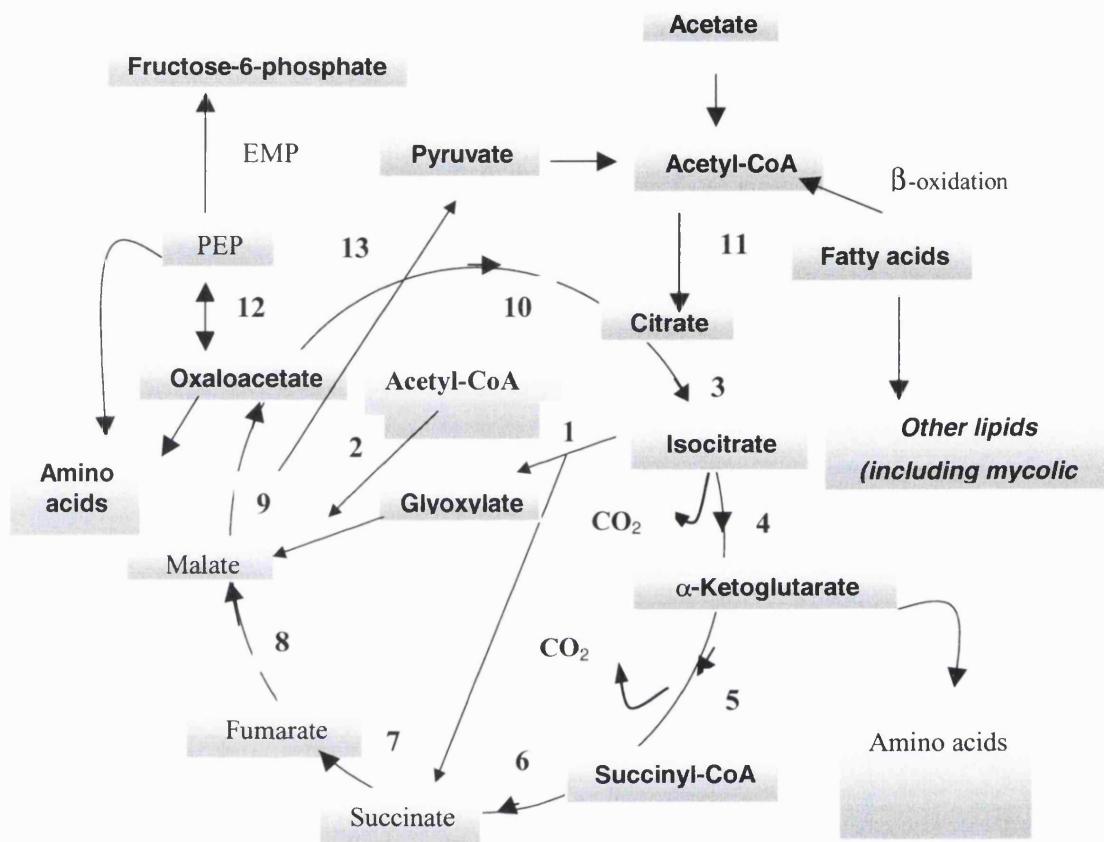


Figure 1.4: The glyoxylate shunt and TCA cycles and related reactions.

Additional routes are shown for the synthesis of metabolites from the products of TCA cycle. In the glyoxylate cycle, isocitrate lyase (reaction 1) converts isocitrate to succinate plus glyoxylate. Malate synthase (reaction 2) then converts glyoxylate plus acetyl-CoA to malate.  $\beta$ -oxidation of fatty acids provides acetyl-CoA for these reactions and using this pathway bacilli bypass two oxidative steps of the TCA cycle.

The enzymes of TCA and glyoxylate cycle are as follows: 1, isocitrate lyase; 2, malate synthase; 3, aconitase; 4, isocitrate dehydrogenase; 5,  $\alpha$ -ketoglutarate dehydrogenase; 6, succinyl-CoA synthetase; 7, succinate dehydrogenase; 8, fumarase; 9, malate dehydrogenase; 10, citrate synthase; 11, pyruvate dehydrogenase complex; 12, phosphoenolpyruvate (PEP) carboxykinase; 13, malic enzyme. EMP Embden-Meyerhof-Parnas pathway.

In addition to the production of isocitrate lyase, there is other experimental evidence that support the view that MTB are metabolically active during latent infection.

Shiftdown of *M. smegmatis* into non-replicating stages on glycerol depletion has been reported recently (Marjan, *et al.*, 1999). Clumping of cells during stationary phase and on prolonged starvation suggests that clumping promotes stationary phase survival. It has been reported recently that cell wall thickening occurs in static, O<sub>2</sub>-starved MTB and *M. bovis* (BCG) cells (Cunningham and Spreadbury, 1998) and this stabilises the cell to face adverse conditions. Marked thickening of the cell wall outer electron opaque layer may influence the clumping of cells. Hence, cells can stick to each other and survive using the cellular content of the dying neighbour cells in the clump before they leak into the environment. This suggests that mycobacteria are able to sense low levels of a range of nutrients and start to adapt to stationary phase well before depletion, using the energy derived from the remaining nutrients in the adaptation process. However, whether stationary phase cultures of MTB are similarly dynamic remains to be demonstrated.

The question of transcriptional activity of MTB during persistence has recently been documented (Pai, *et al.*, 2000). Direct RT-PCR for the mRNA of antigen 85B (AgB) of MTB detected a signal among multiple tissues, from 'sterile' state mice from the Cornell model, which were negative for culture. These observations suggest that bacilli during latent infection are metabolically active.

### **1.7 Immunopathology of latent infection**

In recent years, latent mouse models have been extensively used to study the host factors that are involved in survival of MTB during latent tuberculosis infection. Using either gene knockout mice or neutralisation with antibodies, involvement of various cytokines and cell types in the control of MTB infection have been demonstrated

As the reactivation of latent infection is associated with immunosuppression (Collins, 1989, Rook, 1994)[Sheridan, 1994 #767, latency appears to be

sustained to a certain extent by the physiological condition related to immune mechanisms of the host.

After evading the mechanical defences of the upper respiratory system, droplet nuclei containing viable MTB reach the distal region of the lung where they are taken up by alveolar macrophages, representing the first host-parasite interaction. There may be people in whom macrophages are capable of killing and eliminating the bacilli completely, never allowing a latent stage to develop. However, in those who develop latent infection host immune response is initially compromised by the inability of macrophages to destroy the inhaled mycobacteria. This inability appears to be a result of physicochemical properties of the mycobacterial cell wall, which play an important role in the mode of entry of MTB into the macrophages, its ability to survive within the macrophages and the suppression of the induction of immune response of the host. Nevertheless, the infected macrophages use various mechanisms to kill the intracellular MTB and at the same time induce the immune response of the host.

### **1.7.1 Binding and uptake of *M. tuberculosis* by macrophages**

Mycobacteria are taken up by macrophages through specific interactions between the cell wall components of the MTB and receptors found on the surface of macrophages. MTB can gain entry into macrophages in several distinct ways. The precise route of entry is likely to determine the ultimate fate of the bacilli within the macrophages. MTB can bind to macrophages directly via complement receptors and the macrophage mannose receptors (MMRc) mediated by mannose-capped lipoarabinomannan (man Lam) (Fenton and Vermeulen, 1996). This accounts for only a portion of total phagocytosed MTB. Binding of MTB to macrophages can also be accomplished by surfactant receptors that serve as an opsonin for inhaled organisms and scavenger receptors (Schluger, 2001). Recent studies have demonstrated involvement of toll-like receptors (TLRs) in mediating the uptake of MTB by macrophages. CD14, another receptor on the surface of macrophages binds to LAM of MTB and recruits LAM to TLR, thereby facilitating optimal signal transduction and production of IL-12, resulting in increased production of nitric oxide, which is an important step in intracellular killing of MTB (Modlin, *et al.*, 1999).

Although it was believed that MTB infect and multiply within macrophages, recent findings raise questions about this conventional theory. MTB DNA has been demonstrated in non-professional phagocytes in the lungs of people who died due to causes other than TB, i.e, latently infected people (Hernandez-Pando, *et al.*, 2000). There are also *in vitro* studies that demonstrate entry and multiplication of mycobacteria into non-professional phagocytic cells such as alveolar epithelial cells and endothelial cells (Bermudez, 1991, Bermudez and Goodman, 1996), suggesting involvement of other mechanisms of entry into cells that do not have the receptors that are found on macrophages.

### **1.7.2 Microbicidal action of macrophages**

Killing of ingested MTB is most likely to take place within macrophage phagolysosomes. Toxic constituents of the acidic vesicles include lysosomal hydrolases; reactive oxygen intermediates (ROI) such as  $H_2O_2$  and  $O_2^-$ ; and reactive nitrogen intermediates (RNI) such as NO and  $NO^-$  which are potent killers of intracellular pathogens.

Another postulated defence against this intracellular infection is destruction of infected macrophages via macrophage apoptosis. Apoptosis is a programmed cell death, which prevents the release of intracellular components and the spread of mycobacterial infection by sequestering the pathogens within apoptotic bodies. It is reported that TNF-induced apoptosis of infected macrophages substantially reduce the bacterial burden while non-apoptotic cell death has no effect on the bacterial burden. Apoptosis induced by ATP promotes killing of virulent MTB within human macrophages (Kusner and Adams, 2000) as does apoptosis induced by fas ligand (FasL) (Oddo, *et al.*, 1998). Fas ligand is a member of TNF receptor family, and the binding of FasL to Fas found in macrophages, transduces signal into cell, which will lead to apoptosis of the infected cell. Hydrogen peroxide-induced apoptosis also causes microbicidal effects (Rook, *et al.*, 2001)

Besides destroying invading organisms, one of the main functions of the macrophage is to initiate a full immune response. Immunity to MTB infection is T-

cell mediated. Both CD4 and CD8 lymphocytes are important in controlling the growth of tubercle bacilli. Macrophages induce the host immune response by shuttling peptides from ingested bacteria to the macrophage cell surface and presenting them to T cells (CD4 and CD8 T lymphocytes). The helper CD4 T cell, however, will only recognise an immunogenic peptide if it is presented in the context of major histocompatibility complex (MHC) class II molecules (Pancholi, *et al.*, 1993). Mycobacteria appear to be able to prevent MHC class II molecules from migrating to the macrophage cell surface and altering T cell recognition. They do this by inhibiting the correct maturation and evolution of some of the proteins of MHC class II.

On recognition of the peptides, CD4 cells produce a variety of cytokines, which initiate a cascade of immune responses to the antigen. The production of different cytokines by CD4 cells has led to their classification as either Th1 (dominated by IL-2, IL-12, IFN- $\gamma$  and TNF- $\alpha$ ) or Th2 cells (dominated by IL-4, IL-5, and IL-13).

Protective immunity to tuberculosis correlates with the Th1 response. Th1 cells secrete interferon- $\gamma$  (IFN- $\gamma$ ) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). IFN- $\gamma$  is produced by CD4+ and CD8+ cells and by alveolar macrophages via an IL-12 - dependent pathway. IL-12 and IL-18 have an essential role in the immune response to mycobacteria. Both contribute to the induction of IFN- $\gamma$  expression.

To date, IFN- $\gamma$  knockout mice are the most susceptible to virulent MTB, indicating its protective role in infection. They are unable to control the growth of MTB and although granulomas form in these mice they quickly become necrotic. Macrophage activation is defective and fails to express inducible nitric oxide synthase (iNOS2) (Flynn, *et al.*, 1993). Humans with defective genes for IFN- $\gamma$  or the IFN- $\gamma$  receptors are also found to be prone to mycobacterial infections [Flynn, 2001 #296. IFN- $\gamma$ , which is the only known mechanism that can kill intracellular MTB, governs an essential pathway that activates macrophages and induces iNOS2, the enzyme responsible for NO production from L-arginine. The precise mechanism(s) by which NO and other RNI antagonise MTB is not known, but may involve disruption of bacterial DNA, proteins, signalling and/or induction of

apoptosis of macrophages that harbour mycobacteria (Chan, *et al.*, 2001). Even though there are controversial views about the role of RNI in human tuberculosis infection, a growing body of evidence suggests that NO may be released by human cells (Nozaki, *et al.*, 1997, Jagannath, *et al.*, 1998, Wang, *et al.*, 1998)[Chan, 2001 #141.

All the above-described studies reveal that IFN- $\gamma$  is important for protective immunity against TB. Nevertheless, it does not eliminate the organism completely from the body. In a plateau phase of infection in a murine model, although IFN- $\gamma$  and iNOS2 were produced throughout the plateau phase, inhibition of iNOS2 resulted in reactivation of infection (Flynn, *et al.*, 1998). Therefore, continuous macrophage activation and RNI production is important in preventing reactivation in the lungs but not effective enough to eliminate the organism completely. The role of IFN- $\gamma$  in latent infection is not clear. When the CD4 Cells were depleted in a plateau phase mouse model, it resulted in reactivation of the disease even in the presence of IFN- $\gamma$  gene expression, suggesting that IFN- $\gamma$  alone cannot prevent reactivation (Scanga, *et al.*, 1999). In another study, treatment of the Cornell model mice with anti- IFN- $\gamma$  antibody did not result in reactivation of latent infection (Scanga, *et al.*, 1999).

The role of TNF- $\alpha$  is crucial in the pathogenesis of tuberculosis. MTB induces TNF- $\alpha$  secretion by macrophages, dendritic cells, and T cells. This cytokine is very important for effective granuloma formation and to control bacterial growth at the site of infection (Rook, *et al.*, 2001). It also activates macrophages and induces RNI production. The amount of TNF- $\alpha$  in the lungs during infection determines whether the cytokine is protective or destructive (Bekker, *et al.*, 2000). While high level of TNF- $\alpha$  causes excessive pathology, deficiency resulted in destructive pathology. Toxicity of TNF- $\alpha$  varies according to the presence of other cytokines. Increase in the toxicity of TNF- $\alpha$  has been reported in the presence of IL-4 and IFN- $\gamma$  (Hernandez Pando, *et al.*, 1997).

Neutralisation of TNF- $\alpha$  with anti- TNF- $\alpha$  antibody in mice, that have previously been infected and had stable bacterial load, resulted in destructive and aberrant pathology, disorganised granulomas, diffuse cellular infiltration and squamous

metaplasia in the lungs (Mohan, *et al.*, 2001). Such an aberrant pathology in the absence of TNF- $\alpha$  suggests that this cytokine plays an important role in preventing reactivation of latent TB and limits the pathological response of the host. Neutralisation of TNF- $\alpha$  in a subset of Cornell model mice also resulted in reactivation of infection (Scanga, *et al.*, 2000). Interestingly, reactivation of TB has been reported in a number of patients who received infliximab, a humanised monoclonal antibody against TNF- $\alpha$ , used to treat rheumatoid arthritis and Crohn's disease (Keane, *et al.*, 2001).

These observations have revealed a major role for TNF- $\alpha$  in the control of persistent infection and modulation of the pathogenic response to MTB. It is clear that TNF- $\alpha$  affects cell migration to and localisation within tissues in MTB infection, but its contribution towards maintenance of chronic infection remains to be determined.

IL-12 and IL-18 have an essential role in the immune response to mycobacteria (Sugawara, *et al.*, 1999). Both contribute to the induction of IFN- $\gamma$  expression, and are therefore involved in protective immunity. IL-12 is a crucial cytokine controlling MTB infection. *IL-12p40* gene-deficient mice are quite susceptible to infection and had decreased survival times in comparison with control mice due to their substantial reduction in IFN- $\gamma$  production (Cooper, *et al.*, 1997). Humans with mutations in *IL-12p40* or the IL-12 receptors are also susceptible to mycobacterial infection, disseminated BCG infection, and *M. avium* infection (de Jong, *et al.*, 1998). However, its role in chronic infection is not clear. Nevertheless, administration of IL-12 DNA to mice with chronic infection was found to substantially reduce the bacterial burden in these mice (Lowrie, *et al.*, 1999).

CD8 T cells are also required for immunity against TB, but the relative importance of CD8 T cells is controversial (Mogues, *et al.*, 2001). Most MTB-specific CD8 cells recognise their antigens in association with MHC class I. Lytic peptides secreted by CD8 T cells, granulysin and perforin, have recently been shown to be associated with direct cytolytic activity against MTB (Stenger, 2001). Although CD4 T cells are believed to be the major producers of IFN- $\gamma$  in response to MTB

infection during the early phase of infection, as the infection progresses other cells, notably CD8 T cells also produce this cytokine and add to the overall IFN- $\gamma$  level in CD4-depleted mice. Antibody-mediated depletion of CD4 T cells does not impair the level of IFN- $\gamma$  and iNOS2 production or activity in a plateau phase mouse model, indicating macrophage activation even during CD4 T cell deficiencies (Scanga, *et al.*, 2000). The CD8 T cell population produces more IFN- $\gamma$  in CD4 T cell-depleted mice, as in the acute infection model. Nevertheless, depletion of CD4 T cells results in rapid reactivation of the infection, even in the presence of similar levels of IFN- $\gamma$  found in the control mice. This indicates that CD4 T cells have a role in the control of latent TB infection that is independent of IFN- $\gamma$  and RNI production. The importance of CD4 T cells in controlling latent tuberculosis infection has long been recognised in humans, as the incidence of reactivation of TB is high in AIDS patients.

In contrast to the results of the study described above, in another study of the mouse model, anti-CD8 antibody treatment during the plateau phase of the infection resulted in reactivation while anti-CD4 antibody treatment induced only a modest change in the bacterial burden in the lung (van Pinxteren, *et al.*, 2000). This study showed changes in the role of CD4 and CD8 T-cells over the course of the infection process from acute to latent infection. While the CD8 subset played a relatively modified role during primary infection, it was the most important subset, producing a large amount of IFN- $\gamma$ , maintaining the bacterial burden during the plateau phase of the infection. This indicates that there should be a different set of antigens expressed during the plateau phase of the infection by MTB, and that these could be presented via the MHC class 1. There is a body of evidence that suggests changes in metabolism and expression of different antigens when the mycobacteria are in a non-replicating state as compared to actively growing bacteria (Wayne and Sramek, 1979, Cunningham and Spreadbury, 1998, McKinney, *et al.*, 2000). However, the relationship of these phenomena to true latency remains elusive, as the metabolism and the gene expression of the bacilli in well-oxygenated areas of the lung during latent infection are not known.

While a Th1 response contributes to protective immunity, the Th2 response correlates with non-protective immunity to TB and undermines the efficacy of the Th1 response. Recently, the negative role of Th2 cytokines in human TB has been established (Seah, *et al.*, 2000, van Crevel, *et al.*, 2000). Expression of IL-4 is increased and correlates with severity of disease and with cavitation. Furthermore, IL-4 increases the toxic effect of TNF- $\alpha$  (Hernandez Pando, *et al.*, 1997). During active infection, selective apoptosis of Th1-like T cells may be a scenario that down-regulates the Th1 response (Varadhachary and Salgame, 1998, Das, *et al.*, 1999).

### **1.7.3 Survival mechanisms of *M. tuberculosis* within the macrophage**

Establishment of latent infection demands survival of MTB despite the strong immune response that the host exerts to eliminate it. Clearly, the host response is effective in containing the bacilli, since one-third of the world's population harbour the bacilli without any clinical symptoms and a slight imbalance in the immune system can result in reactivation of the latent infection. The ability of the bacilli to survive the robust response clearly implicates a series of evasive mechanisms.

#### **1.7.3.1 Evasion of phagolysosome fusion**

The macrophage mannose receptors (MMRc) mode of entry seems to bypass the normal macrophage ingestion process, thereby avoiding the 'respiratory burst' (Pabst, *et al.*, 1988, Chan, *et al.*, 1991). Respiratory burst is a process that produces reactive oxygen intermediates which, along with toxic nitrogen intermediates and a cocktail of proteinases and other enzymes, are used to acidify the lysosomes (vacuoles) within the macrophages, which usually fuse with the phagosome, and kill any ingested bacteria. A capacity to block the fusion of mycobacterium-containing phagosomes with lysosomes is critical for the survival and replication of MTB in infected human macrophages (Clemens and Horwitz, 1995). An electron microscopic study has also shown budding of vesicles containing MTB from phagosomes with no subsequent fusion with lysosomes (McDonough, *et al.*, 1993). An interesting recent study also showed a possible phenomenon that bacilli might use to survive within the macrophages. This study

demonstrated accumulation of cholesterol at the site of phagocytosis in macrophages and depletion of cholesterol dramatically impaired the mycobacterial uptake (Gatfield and Pieters, 2000). Cholesterol also mediated the phagosomal association of TACO. TACO is a coat protein found on MTB infected macrophages and shown to prevent mycobacterial phagosome maturation and into or fusion with lysosomes. This suggests that by entering host cells at cholesterol-rich domains of the plasma membrane, mycobacteria may ensure their subsequent survival in TACO-coated phagosome by inhibiting lysosomal degradation.

#### **1.7.3.2 Negative regulation of macrophages**

Prevention of lysosome acidification also seems to involve protein phosphorylation-mediated communication (a cellular signalling mechanism used by many cells) between the mycobacteria and macrophages. Two protein kinases, tyrosin kinase and serine/threonine kinase, have so far been identified as potentially being involved in this signalling mechanism (Nandan, *et al.*, 2000).

Lipoarabinomannan (LAM) from MTB, one of the chemically defined virulence factors, plays an important role in blocking macrophage activation and sustaining intracellular survival of bacilli. LAM is capped with a mannose residue, which inhibits both tyrosine phosphorylation in the macrophage and increases the activity of the phosphotyrosine phosphatase, SHP-1, an enzyme that dephosphorylates tyrosine residues, thereby disrupt macrophage signal transduction (Nandan, *et al.*, 2000) and impair T cell recruitment.

#### **1.7.3.3 Inhibition of macrophage apoptosis**

As described under 1.7.2, certain types of apoptosis appear to reduce the viability of the contained mycobacteria. It has been noted that the release of soluble type 2 TNF receptors (sTNFRII) induced by virulent strains of MTB may limit the apoptotic death of infected alveolar macrophages and that they also have reduced Fas expression which may limit this pathway of apoptosis induction (Oddo, *et al.*, 1998).

#### 1.7.3.4 Negative cytokine function and latency

MTB induces macrophages to produce IL-10 and transforming growth factor  $\beta$  (TGF $\beta$ ), which are anti-inflammatory and immunosuppressive cytokines that deactivate macrophages. TGF $\beta$  enhances IL-10 production in human monocytes and may interact synergistically with IL-10 during TB infection (Maeda, *et al.*, 1995). Recently a T cell clone with a cytokine profile different from that of Th1 and Th2 has been reported. This subset of cells has been termed T regulatory type 1 cells. These cells were also shown to produce IL-10, and thereby inhibit the antigen-specific activation of naïve T cells (Levings, *et al.*, 2001).

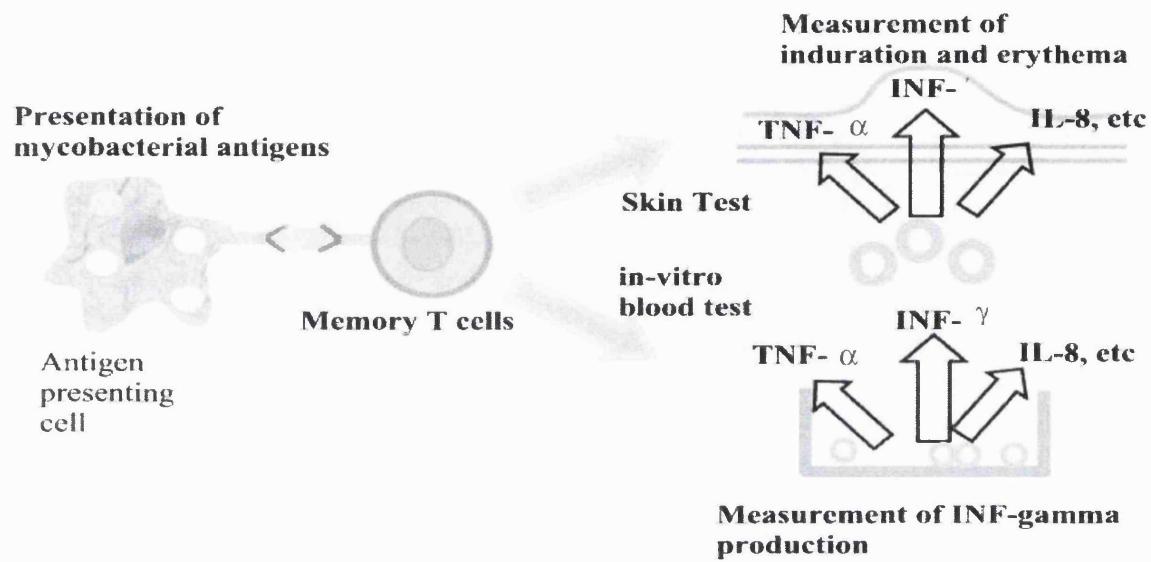
IL-10 can inhibit the production of many cytokines of the Th1 response including IL-2, IFN- $\gamma$  and TNF- $\alpha$ , thereby deactivating macrophages. Various surface molecules are also down regulated by IL-10 including MHC class II. Production of ROI and RNI in activated macrophages and macrophage-dependent T-cell proliferation are also inhibited (Redpath, *et al.*, 2001). IL-10 was also shown to prevent TNF- $\alpha$  mediated apoptosis (Rook, *et al.*, 2001). A recent study has demonstrated an essential role of IL-10 during the latent phase of infection with *Leishmania*. Neutralisation of IL-10 in the persistence of *Leishmania major* infection resulted in complete eradication of the organism from healed skin lesions (Belkaid, *et al.*, 2001). From these observations: it is tempting to hypothesise that IL-10 production induced by MTB may reduce the inflammatory response and may thereby suppress the development of a hostile environment for the bacilli. During this scenario, bacilli can adapt themselves and lurk in the body for decades.

### 1.8 Diagnosis of latent tuberculosis infection

During the last decade tuberculosis case rates have significantly declined in industrialised countries. Nevertheless, elimination of tuberculosis will only be a realistic goal if latently infected people are identified and treated. This can only be achieved with an efficient diagnostic method that can detect latently infected people.

Various methods are available to detect active infection including sputum microscopy and culture. Specificity of microscopic examination of sputum for acid-fast bacilli varies from 22% to 65% (Salfinger and Pfyffer, 1994). Culture of sputum however increases this proportion to 80%. In recent years, *in vitro* amplification of mycobacterial target DNA by PCR in the sputum has been extensively exploited as a diagnostic method for tuberculosis. However, these methods seem to be less sensitive with smear negative sputum compare to smear positive sputum (Bergman, *et al.*, 1989, Carpentier, *et al.*, 1995). Therefore, none of these methods can be used to detect latent infection, as the infected individuals do not produce sputum.

Methods that are in use today to detect LTBI are immune-based (Fig 1.5). An immune-based test with specific antigens should be able to detect whether a person's immune system is sensitised by MTB and thereby confirm infection. Nevertheless, one does not need live bacilli to persist to retain a strong state of memory immunity (Andersen, *et al.*, 1995). Memory cells are long-lived and they can be continually re-stimulated by specific antigens. Hence, positive reaction for an immune-based test does not necessarily mean that the person is latently infected.



**Figure 1.5: *In vivo* and *in vitro* diagnostic tests**

Both *in vivo* (skin test) and *in vitro* (blood test) depend on the elaboration of inflammatory cytokines and T cells previously sensitised to mycobacterial antigen. In the skin test, mycobacterial antigens are injected below the epidermal layer, causing infiltration of antigenic-specific lymphocytes and the elaboration of inflammatory cytokines. This inflammatory reaction results in the characteristic indurated area at the site of injection. In the blood test, mononuclear cells from the peripheral blood are stimulated *in vitro* and production of IFN- $\gamma$  from sensitised T cells is measured by ELISA. (Adapted from (Andersen, *et al.*, 2000)).

### 1.8.1 Tuberculin skin test (TST)-*in vivo* test

Until recently the tuberculin skin test was the only proved method for identifying infection with MTB in persons who do not have TB disease.

#### *Immunological basis for the tuberculin reaction*

Reaction to tuberculin is a sign and measure of immunity. In the tuberculin skin test, a known quantity of purified protein derivative (PPD); tuberculoprotein, precipitated with acetone and ammonium sulphate, is injected intradermally into either the dorsal or volar surface of the forearm. After intradermal tuberculin challenge, if the person is already sensitised a type IV hypersensitivity occurs. Antigen presenting cells (APC), macrophages, and dendritic cells present the antigen to T cells. Following earlier priming, memory CD4 T cells recognize the antigen together with class II Major histocompatible complex (MHC), on these APC and release Th1 cytokines (Tsicopoulos, *et al.*, 1992). These cytokines activate macrophages to accumulate and form a granuloma at the injection site. Different sized nodules appear around the injection site after 24 hours and peak at 48-72 hours.

For persons with latent infection and normal immune responsiveness, test sensitivity approaches 100% (Rose, *et al.*, 1995). The main shortcoming of this method is cross reactivity of the antigens present in PPD. Some antigens present in PPD are shared with other mycobacteria and therefore tuberculin reactivity can result from immunization with BCG or from exposure to environmental mycobacteria (Sepulveda, *et al.*, 1990, Comstock, 1994). These false positive reactions result in a lower specificity and a low positive predictive value in persons who have a low probability of latent infection. Another draw back of this test is false-negative reactions (anergy) that can occur in patients with concurrent infections (especially measles and HIV infection), in those who are immunosuppressed, in the elderly, and in those with particular genetic features associated with HLA-D (class II) histocompatibility complex haplotypes (Rodysill, *et al.*, 1989, Cainzos, *et al.*, 1993, Maas, *et al.*, 1999). Simultaneous anergy testing with the tuberculin test can reduce false positive reactions. However, utility of anergy testing together with tuberculin reaction has shown that reactivity

to the control antigens did not predict tuberculin reactivity (Yanai, *et al.*, 1997). Immune response to tuberculin and control antigens (candida and mumps) was tested in HIV patients. While HIV positive people with a high CD4 count reacted markedly less to tuberculin, reactivity to mumps and candida was present in more than half of HIV positive persons with a low CD4 count, suggesting anergy testing has a limited role in identifying LTBI in HIV infected people.

Specificity of the tuberculin skin test is also dependent on the criterion used to define a “positive” test. The specificity can be improved by progressively increasing the reaction size that separates positive from reactors (at the expense of decreasing test sensitivity) (Rose, *et al.*, 1995).

### **1.8.2 Interferon gamma assay-*in vitro* diagnostic test**

This is a blood test, based on the production of interferon gamma by the mononuclear cells in response to mycobacterial antigen. In this assay, whole-blood cells are stimulated with the antigen for 24 hours, followed by ELISA detection of IFN- $\gamma$ .

The only commercially available blood test to detect LTBI is Quantiferon-TB test (CSL Biosciences, Melbourne, Australia). In this test, production of interferon gamma by mononuclear cells in response to MTB is compared with IFN- $\gamma$  production in response to predominant environmental mycobacterium, *M. avium* complex. Results of this test have shown that whole-blood IFN- $\gamma$  production from healthy individuals not vaccinated with BCG stimulated overnight with MTB PPD *in vitro* correlated well with TST (Pottumarthy, *et al.*, 1999). In a comparative study of TST and IFN- $\gamma$  assay, it was found that the odds of having TST positive but IFN- $\gamma$  negative were 7 times higher for BCG-vaccinated people compared to unvaccinated people, indicating that IFN- $\gamma$  is less affected by BCG vaccination. It also provided evidence of responses due to nontuberculous mycobacteria among unvaccinated people (Mazurek, *et al.*, 2001). In addition, within a population of intravenous drug users, in whom immunosuppression is commonly a confounding factor, the IFN- $\gamma$  whole-blood test detected higher numbers of PPD-positive individuals than the TST test (Converse, *et al.*, 1997). However, multiple MTB

antigens used in this test also will result in a false positive reaction in BCG unvaccinated individuals (Brock, *et al.*, 2001).

### **1.8.3 Whole-blood interferon gamma response to MTB specific antigens**

During the past decade, many antigens that are expressed by MTB, but not by BCG or by most environmental mycobacteria, have been identified (Andersen, *et al.*, 2000). Among these specific antigens, genes for ESAT-6, CFP10, and MPT64 were found to be deleted in *M. bovis* BCG during *in-vitro* passage (Mahairas, *et al.*, 1996). In recent years, application of these antigens in whole-blood IFN- $\gamma$  assays has been extensively exploited to utilise them in diagnosis of TB and LTBI (Arend, *et al.*, 2000, Brock, *et al.*, 2001). The best studied among these antigens is the early secreted antigen target 6-kD protein (ESAT-6) (Arend, *et al.*, 2000, Lalvani, *et al.*, 2001c) and this has been shown to be an attractive candidate to use in LTBI diagnosis. Interferon gamma production by memory T cells in response to ESAT-6 was measured by enzyme-linked immunospot (ELISPOT) in TB patients; household contacts and BCG vaccinated and unvaccinated individuals (Lalvani, *et al.*, 2001b). In this study, 85% of 26 people presumed to have LTBI (based on a positive Heaf test) were positive by the ELISPOT test. All the BCG-vaccinated unexposed controls were negative. This specificity confers a major advantage of this method over others that are currently in use to detect LTBI.

### **1.8.4 Chest Radiographs**

Chest radiography is also currently used in screening. In persons with latent infection, the chest radiograph is normal, although it may show abnormalities suggestive of prior TB. Nodules and fibrotic lesions of previous, healed TB have well-demarcated, sharp margins. These scars may contain non-replicating tubercle bacilli with substantial potential for future progression to active disease.

## 1.9 Treatment of latent tuberculosis infection

TB is, basically, a treatable disease. WHO claims a cure rate of up to 95% with multiple drug therapy such as DOTS (Directly Observed Treatment-Short-Course) (Choi, 1998). DOTS involves a regimen of four first line antimycobacterial drugs: isoniazid, rifampicin, pyrazinamide and ethambutol (or streptomycin), taken intensively for two months, followed by further a four months of isoniazid and rifampicin.

Tubercle bacilli in latent infections are not necessarily genetically resistant to the antimycobacterial drugs, as it has been noted they were sensitive to the same drugs when they are recovered and grown *in vitro* in nutrient media. Wayne and Hayes noted the bactericidal effect of metronidazole, a drug commonly used for anaerobic bacteria, on the NRP 2 stage (Wayne, 1994). Recently, Mitchison and colleagues have studied the activity of the metronidazole antibacterial effect in the Cornell model of murine TB and concluded that the drug has no effect in the initial sterilising phase or subsequent sterile state (Dhillon, *et al.*, 1998). They suggested that the oxygen tension in the cellular lesions of murine tuberculosis is unlikely to be sufficiently low to allow metronidazole to act.

In recent years, interest has been focused on designing drug regimens to treat latent infection, especially, in HIV patients who are at high risk of developing reactivation of endogenous infection. New recommendations for "Targeted Tuberculin Testing and Treatment of Latent Tuberculosis Infection" were published recently by the American Thoracic Society (2000). Preventive therapy with either twice-weekly isoniazid for 6 months or a combination of rifampicin and pyrazinamide for 3 months reduced the incidence of TB in HIV-infected persons (Mwinga, *et al.*, 1998). However, this preventive therapy was effective in people with less advanced immunosuppression (people with positive tuberculin skin test or a leukocyte count of  $2 \times 10^9/l$  or greater). Furthermore, analysis of rifapentine for protective therapy in the Cornell model of latent tuberculosis showed that once-weekly isoniazid plus rifapentine combination therapy for 18 weeks was an effective preventive therapy regimen with sterilising potency and bacillary load

reduction comparable to those of daily isoniazid therapy for 18 weeks (Miyazaki, *et al.*, 1999).

The recommendation of treatment of latent infection with the drugs that are available to treat active infection is confusing. All the antimycobacterial agents currently in use to treat TB affect only actively growing MTB, as their main targets are enzymes involved in cell envelope biosynthesis. Therefore, they are relatively ineffective against the bacilli lurk in the body during latent infection.

Findings of cell envelope thickening during oxygen starvation (Cunningham and Spreadbury, 1998) support the resistance of non-replicating bacilli during latent infection to antimycobacterial agents. Other, recent findings have highlighted a protein, a resuscitation promotion factor, involved in the reactivation of latent mycobacteria (Mukamolova, *et al.*, 1998). Various other proteins such as isocitrate lyase and alternative sigma factors were also found to be involved in the persistent state of the bacilli (Michele, *et al.*, 1999, McKinney, *et al.*, 2000). Thus, development of drugs that can inhibit the enzymes, which are important for mycobacterial persistence in the host would block the development of reactivation of latent infection.

Hence, an improved understanding of the mechanism of mycobacterial latency and endogenous reactivation would significantly contribute to the control of TB.

A number of questions need to be addressed to understand the mechanism of MTB in latent infection, which include:

1. Are the latent bacilli in an altered developmental state in which they are non-acid fast?
2. What is/are the specific site(s) of latency and /or persistence of bacilli, in terms of both organ and cell type(s)?
3. Do the latent bacilli exist in a spore-like state or metabolically active stationary-growth phase?
4. How long will the cell carrying the bacilli survive and how will the bacilli find and infect another cell after the death of the original cell?

5. What triggers or facilitates the conversion of actively growing bacilli into a latent state and reactivates the dormant bacilli?
6. What are the genes involved in the regulation?

The major aims of this project are to develop an efficient method to detect latently infected people, to study the organ and cellular location of *Mycobacterium tuberculosis* during latent tuberculosis infection, and to attempt to study the morphology of the bacilli during latent infection.

## Chapter 2

### 2. Materials and Methods

The composition of solutions and details of suppliers are listed in the appendix 1 and 2, respectively.

#### 2.1 Microscopy

Both Ziehl Neelsen (ZN) and fluorescence microscopy (Collins, 1997) were used to examine sputum smears and tissue sections.

##### Preparation of samples for microscopy

###### *(a) Liquefaction and smear preparation of sputum samples for microscopy*

An equal volume of 2% sodium hydroxide-N-acetyl-L-cysteine solution was added to the sputum and mixed well by vortex mixing periodically for 20 minutes at room temperature. Dissolved sputum was then transferred to disposable universal containers and centrifuged at 3,700 g for 15 minutes. Supernatant was discarded and the pellet was re-suspended in excess phosphate buffer (ph 6.8) and centrifuged for 15 minutes at 3,700 g. The pellet was used to prepare smears for microscopy and for culture. Any remaining pellet was re-suspended in 1 ml molecular biology grade water (MB water) (Sigma) and stored at -20°C until use for DNA extraction. Sputum samples were processed in a class 1 cabinet in a category 3 laboratory.

###### *(b) De-paraffinisation of tissue sections*

Paraffin tissue sections were deparaffinised at 60°C for 15 minutes, followed by two 3 minutes washes with xylene. Sections were re-hydrated through 100, 96 and 70% absolute ethanol and finally in water.

#### 2.1.1 Auramine staining

Sputum smears were heat dried on a heating block prior to staining. Tissue sections and sputum smears were first stained with Auramine (Sigma) for 10 minutes. Slides were washed in tap water and decolourised with acid-alcohol for 5 minutes. After washing with tap water, sections and smears were

counterstained with 0.1% KMnO<sub>4</sub> for 30 seconds. Extra stain was removed by washing and the slides were allowed to air dry. Tissue sections were dehydrated through graded alcohol before examination under the high power (x100) oil immersion lens.

### **2.1.2 Ziehl Neelsen staining**

The rationale behind this staining method is that the phenol present in the carbolfuchsin solution enables the dye (basic fuchsin) to penetrate the lipid coat of the mycobacteria, thus staining them sufficiently to allow subsequent differentiation and visualisation. The organisms then display acid-alcohol fastness; the background can be decolourised using 1% acid-alcohol, and then counterstained with a green dye (malachite green) for maximum contrast.

A cold acid-fast staining method was employed. Briefly, sections and smears were first treated with carbolfuchsin followed by decolourisation with acid-alcohol and counter stained with malachite green.

After staining, more than 20 fields of each stained smear were examined carefully under the light microscope using an oil immersion (x100) lens.

## **2.2 Culture**

Sputum samples were processed as for staining, and inoculated into Lowenstein –Jensen (LJ) slopes and incubated at 37°C. The slopes were examined for growth every week for 12 weeks.

## **2.3 Indirect Enzyme Linked Immunosorbant Assay (ELISA)**

The indirect ELISA assay allows the detection and/or the titration of specific antibodies bound to a given unlabelled antigen immobilised on a micro-titration ELISA plate. Bound antibodies are specifically detected using an enzyme-conjugated to a molecule specific for the bound antibody. Enzymes such as peroxidase and phosphatase are often used. In the final stage, a chromogenic substrate is added, which generates a coloured product in the presence of the

enzyme portion of the ligand. The optical density of this solution is measured after a defined period. This is proportional to the amount of the enzyme, which is in turn related to the amount of test antibodies. From this the amount of antibody may be calculated.

The optimal working dilutions were determined by checkerboard titration. Two wells were used in each plate as a control for non-specific binding (with antigen, conjugate and substrate). Test sera were also incubated in wells without antigens in order to ascertain the level of background binding. Background values were subtracted from the mean values for each sample.

One hundred microlitres (100  $\mu$ l) of the 16kDa, 38kDa, 65kDa and 70kDa antigens (Lionex diagnostics and therapeutics) 2  $\mu$ g/ml, 1  $\mu$ g/ml, 1  $\mu$ g/ml, and 2 $\mu$ g/ml respectively, were coated overnight in PBS buffer (pH 7.4), at 4°C, in Nunc-MaxiSorp® microtitre plates (Fisher Scientific UK Ltd). After blocking with PBS/0.05% Tween/5% milk powder (Marvel) for 2 hours at 37°C using 200  $\mu$ l volumes, the contents of each well were then tipped out, the inverted plates banged on tissue paper and washed with PBS/0.05% Tween three times. The samples were then added at 100  $\mu$ l per well at a 1/200 and 1/500 dilution for 16kDa and 38, 65 and 70kDa respectively and incubated at 37°C for 2 h. The plates were then washed three times and incubated overnight at 4°C with rabbit anti-human IgG-horseradish peroxidase (HRP) (Dako), diluted 1:500 for 16kDa and 1:000 for 38, 65 and 70kDa. Following three further washes, bound peroxidase activity was detected with 100  $\mu$ l/well ABTS (Sigma). The enzymatic reaction was stopped by the addition of 100  $\mu$ l of 1% sodium fluoride solution. The colorimetric reaction was measured in a Dynatech MR 5000 ELISA reader at dual wavelengths. Each plate had a positive (a TB positive serum which had given a high reading in the preliminary experiment) and a negative control added in a random order to ensure comparability of results.

The optical density (O.D) for each sample was then corrected for differences between plates by the following calculation:

$$= \frac{\text{O.D sample}}{\text{O.D of the positive control for that plate}} \times 100$$

Sensitivity and specificity results were calculated using the following formulae:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positive} + \text{False negatives}} \times 100$$

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negative} + \text{False positives}} \times 100$$

The cut-off value for ELISA was calculated from the absorbance values from healthy controls by adding two standard deviation to the mean value (Vikerfors, *et al.*, 1993).

#### 2.4 Simple immunoblotting assay

The antigen specific total antibody response was detected in clinical samples (sera and CSF) using a simple immunoblot assay.

A nitrocellulose membrane (BioTrace NT) (Pall Corporation) was coated with a panel of diluted mycobacterial antigens diluted in normal saline (0.9% saline). The membrane was first soaked in normal saline for few minutes and then placed into blotting equipment, the Deca-Probe incubation manifold (Amersham Pharmacia Biotech). This equipment allows screening of 10 samples against 10 antigens at a time. Lanes were marked on the membrane with a pen. Diluted antigen (2 ml) was loaded to each lane of the incubation chamber and left to coat the membrane overnight on a rocker at 4°C. The next day, lanes were washed 6 times with normal saline; each wash lasted for 10 minutes on a rocker. The

membrane was removed from the incubation chamber and transferred to a plastic box containing 2% milk in normal saline where any unbound surface of the membrane was blocked for an hour at room temperature.

The membrane was washed three times in normal saline and replaced into the manifold turning through 90° to the previous position. Lanes were marked with a pen at the centre of each lane at both ends. Using a volume of 2.5 ml, samples (1/400 serum diluted in 0.2% milk/normal saline and neat CSF) were added to each lane. The manifold was incubated overnight at 4°C on a rocker. The next day lanes were quickly washed 10 times with tap water followed by 6 times with 0.2% milk; each wash was for 10 minutes on a rocker.

Two ml of 1:1000 rabbit anti-human IgG, IgM, IgA, kappa, lambda-peroxidase (Sigma) antibody was added to each lane and incubated at room temperature for 2 hours. After incubation, lanes were quickly washed with tap water followed by six washes with 0.2% milk.

The membrane was then removed from the manifold and washed three times in normal saline followed by two washes with distilled water. Finally, substrate, 4-chloro-1-naphthol (Sigma), was added to the membrane and the reaction was visualised.

## **2.5 Isoelectric focusing (IEF)-colorimetric and chemiluminescent methods**

The technique IEF is a combination of electrophoresis and nitrocellulose immunofixation. The principle of the technique is electrophoretic separation of proteins according to their *pI* (isoelectric point). Components that differ by 0.001 pH unit or less can be resolved in a pH gradient set up between a cathode and anode with the cathode at a higher pH than the anode. Proteins migrate until they align themselves at their *pI* where they have no net overall charge and therefore concentrate at this point, where their migration ceases. Then proteins are transferred to a nitrocellulose membrane (NCM) and detected by double antibody and horseradish peroxidase as a visualising agent.

### **2.5.1 Preparation of gel**

IEF was performed using materials and equipment marketed by Pharmacia, UK. Focusing of protein took place on an agarose gel prepared between 24 hours and 7 days in advance and kept at 4°C. To make the gels, 3.6g of D-sorbitol (Sigma) and 0.3g IEF agarose (Amersham Pharmacia Biotech) was weighed and added to 10% 30ml glycerol in a screw-capped bottle and dissolved by heating for 15-20 minutes in a boiling water bath. Meanwhile, the casting frame (Amersham Pharmacia Biotech) for the gel was assembled. A glass plate 127x255mm was overlaid with Gel Bond (ICN, Biomedicals) cut to the dimension of the perspex casting frame and the hydrophobic surface placed downwards onto a pool of 50% methanol in water. Smooth contact between Gel Bond and glass plate was achieved by passing a roller across the surface of the Gel Bond, and squeezing out excess methanol. The Perspex-casting frame was positioned over the top of the Gel Bond and secured by well-positioned bulldog clips. The plate was placed on a levelling table and warmed with a hot air dryer for one minute immediately prior to casting the gel. Before pouring the gel, the agarose solution was allowed to cool to 65°C in a water bath set at 65°C, at which point ampholytes were added: 2ml of pharmalyte 3-10 (Amersham Pharmacia Biotech) and 0.5ml of pharmalyte 8-10.5 (Pharmacia, UK) These amplify the pH gradient, at which range most of the IgG have their *pI* value. The bottle was gently rolled to mix the

ampholytes well (avoiding the creation of air bubbles) and agarose gel solution immediately poured onto the warmed glass plate. Setting took place at room temperature over 15 minutes, after which time the frame was removed and the gel plus Gel Bond plus glass plate transferred to a plastic box, lined with moistened paper towels to prevent the gel drying out, for storage at 4°C.

### **2.5.2 Preparation of nitrocellulose membrane (NCM) for blotting**

Detection of antigen-specific IgG oligoclonal bands was performed with a nitrocellulose membrane (Biotrace *N7*) (Pall Corporation) coated with the antigen under investigation (16kDa-20µg/ml; 38kDa-10µg/ml). Wearing gloves, the membrane was cut to the required size and the appropriate antigen in 0.9% saline was added to the membrane in a plastic box. This was incubated overnight on a rocker at 4°C. Before use, the membrane was washed 3 times in 0.9% saline in non-pyrogenic water. The antigen-impregnated membrane was blocked in 2% dry milk powder (Marvel) in 0.9% saline for 45-60 minutes. If necessary blocked membrane was washed, dried and stored at -20°C until required.

### **2.5.3 Running the gel**

When the gel was required for use, the 2mm meniscus around the gel perimeter was removed by trimming with a scalpel blade. The Gel Bond plus gel face up, was placed carefully across the flat bed electrophoresis cooling plate using a pool of 50% methanol in water spread in between to improve contact between the surface of the tank and Gel Bond. Excess methanol was soaked up from the edges. To improve the evenness of the surface and remove surface fluid, the gel was carefully blotted with a dry nitrocellulose membrane. An application foil (Amersham Pharmacia Biotech) was placed parallel to the lower anodic edge of the gel and any air bubble trapped between the application foil and the gel was released. CSF (5µl) and 1/400 diluted homologous serum samples in distilled water were applied to the successive channels in the foil. The end channels were filled with distilled water. The anodic and cathodic electrode wicks were soaked in the appropriate solutions, any excess electrolyte solution was blotted off and the wicks laid length wise 5mm in from the anodic and cathodic edges of the gel respectively. Excess moisture generated during focusing was removed by

positioning folded paper towels each one just touching the upper and lower edges of the gel. The tank lids were placed over the cooling plate with the appropriate electrodes positioned to make best contact with their representative wicks.

#### **2.5.4 Electrophoresis parameters**

Focusing was performed using a Pharmacia electric power pack at 1250V, 150mA current and 20W power per gel for an integrated period of 1 hour and 20 minutes. Halfway through the electrophoresis the sample application foil was removed, and folded paper towels at the edge of the gel were replaced with new ones. The tank was kept cool throughout by circulating tap water through the tank and performing the experiment in the cold room.

#### **2.5.5 Blotting**

At completion, unfocused surface material was removed by pre-blotting the gel for 30-60 seconds with the nitrocellulose membrane (NCM). For total oligoclonal antibodies, a second sheet of NCM cut to the dimensions of the gel was laid, and for the antigen-specific oligoclonal antibodies, a previously prepared antigen-coated membrane was laid on the focused gel surface, excluding all air bubbles with gloved fingers. This was overlaid with another wet NCM and fine filter paper (Whatman) followed by several paper towels, a glass plate, and weight of 1kg. The gel was thus compressed for 25 minutes on the flat bed electrophoresis cooling plate, with the cold water still circulating to diminish diffusion in the gel.

#### **2.5.6 Immunofixation**

After blotting, the NCM was peeled off from the agarose gel, and washed several times under tap water. The membrane was immersed in 0.2% saline, with the protein side facing up, for 5 minutes on a rocker at room temperature (RT). Immunofixation of the focused IgG transferred to the NCM by blotting, was by use of goat anti-human Fc specific IgG (Sigma) diluted to 1:1000 in 0.2% milk in saline for 1 hour on a rocker at RT, followed by washing with several changes of

tap water. The membrane was then incubated with horseradish peroxidase conjugated rabbit anti-goat IgG (Dako) diluted as the first antibody but for only 30 minutes. Finally, the NCM was washed as above and rinsed in saline three times and then a final rinse with distilled water.

#### **2.5.7 Staining-colorimetric**

50mg of ethyl-amino carbazole (Sigma) was dissolved in 20ml of methanol. 100ml of 0.02M sodium acetate buffer at pH 5.1 was added to this and prior to addition of stain to the membrane, 100 $\mu$ l of 30% (w/w) H<sub>2</sub>O<sub>2</sub> was added. The NCM was incubated in this solution until fully stained (approximately 10 minutes). It was then washed, blotted, and allowed to dry at RT. The membrane was examined for oligoclonal bands under direct light.

#### **2.5.8 Staining-chemiluminescent**

For the chemiluminescent procedure, the blotted membrane was incubated with 1.5000 primary antibody, goat anti-human IgG, for 1 hour on a rocker at RT followed by washing with several changes of 0.2% milk in normal saline (2 quick rinses, 2x15 minutes and 2x5 minutes). The blot was then incubated with horseradish peroxidase conjugated rabbit anti-goat IgG (Dako) diluted 1.5000 for 30 minutes. Finally, the membrane was washed as for the primary antibody and given a final rinse in distilled water.

For chemiluminescent detection, the membrane was incubated for one minute in 20 ml ECL® detection solution (Amersham Pharmacia Biotech) and placed in between two plastic sheets. The membrane was then exposed to X-ray film (Amersham Pharmacia Biotech) in the dark for different lengths of time depending on the intensity of the signal. The film was allowed developed using an auto film developer and the presence of bands was examined over a light box.

Interpretation: IEF patterns are usually interpreted in terms of bands. In an oligoclonal response, which is thought to represent the products of a limited number of clones of plasma cells, multiple bands appear in the sample lane. This is distinct from the polyclonal background, which is the product of a very large

number of plasma cell clones secreting immunoglobulins. The normal amount of polyclonal background IgG is relatively low in CSF as all of the gamma- globulins are derived from serum. Therefore, the presence of oligoclonal bands in the CSF but not in the matched serum indicates intrathecal synthesis (Keir, *et al.*, 1990, Thompson and Keir, 1990). On the other hand, the presence of oligoclonals in the serum alone indicates long-term colonisation by *M. tuberculosis*.

## 2.6 Procedures of Immunohistochemistry

The basic principle of immunohistochemistry is the use of enzyme-linked antibody to detect tissue antigens. The colourless substrate is converted by the enzyme into a coloured product that precipitates on the slide at the site of the reaction. Thus, immunohistochemistry localises antigens in a tissue section. In this study, a cross-reactive *M. bovis* (BCG) polyclonal primary antibody was used to detect MTB cells and cell fragments in tissue sections from TB patients and latently infected persons (Wiley, *et al.*, 1989).

The fixation and staining was performed on microscopic slides pre-treated for maximum cellular adhesion (Merck). Sections (5  $\mu\text{m}$ ) were fixed on the slides and kept at 37°C overnight to allow the sections to adhere to the slides. Sections were deparaffinised by leaving them at 60°C in xylene for 15 minutes. Sections were washed twice in xylene at room temperature (RT) and twice in absolute alcohol to remove the residual xylene. Before staining sections were rehydrated through 96% and 70% ethanol: 3 minutes in each and then left in PBS for 30 minutes. Target antigens were retrieved using target retrieval solution (Dako). For this, slides were placed in plastic Coplin jars filled with target retrieval solution, and heated in a microwave (~700W) at high power for 5 minutes. Slides were then allowed to cool down at RT for 20-30 minutes. Prior to performing antigen antibody reactions, the section area was marked with a PAP pen (Sigma). This forms a barrier around the sections and prevents solutions spreading all over the slide.

Endogenous peroxidase activity in the cells was blocked by a 5-minute incubation with 3% H<sub>2</sub>O<sub>2</sub> (w/w) (Sigma). Then non-specific sites were blocked with 3%

bovine serum albumin (BSA) (Sigma)/PBS for 30 minutes at RT. For staining 100 µl of rabbit anti-*Mycobacterium bovis* (BCG) polyclonals (Dako) diluted 1/1000 in 1.5% BSA/PBS was added to each section and slides were incubated overnight at 4°C in a humid chamber. After three washes with PBS, 100 µl of biotinylated swine anti-rabbit secondary antibody (Dako) diluted 1/500 in 1.5%BSA/PBS was added to each section and incubated at RT for 45 minutes in a humid chamber. Slides were washed three times in PBS and drained before adding streptavidine-HRP (Daco, Denmark) diluted 1/500. Sections were further incubated for 45 minutes at RT with streptavidine-HRP. Slides were washed before visualising superficially bound peroxidase conjugate antigens by using DAB (Dako) substrate. The substrate enzyme reaction was carried out for 8-10 minutes at RT and slides were washed in tap water, and counterstained with haematoxylin (Merck) and dehydrated before microscopic examination.

To evaluate the specificity of staining, normal rabbit serum was substituted for the antimycobacterial antibody as a negative control. A positive control was included in each experiment to ascertain the effectiveness of the reagents used.

## **2.7 Haematoxylin and eosin staining**

Sections were deparaffinised as for immunohistochemistry and stained with Harri's haematoxylin (Merck) for 10 minutes. Slides were washed in water and differentiated in acid alcohol (1% hydrochloric acid and in 70% alcohol) for 10 seconds. After washing the slides in water, sections were stained with eosin for 4 minutes. After a final wash in water, sections were dehydrated and mounted permanently.

## **2.8 Polymerase Chain Reaction (PCR) assays**

PCR is used to rapidly amplify the number of specific DNA sequences in a sample. First, template DNA in the sample to be tested is heated to denature the DNA, higher temperature needed for higher G+C content, so that it will be single stranded. Primers, which are oligonucleotides that will bind to specific sequences of single stranded DNA, anneal to the template DNA if the matching sequences are present. During annealing the reaction mixture is cooled to a temperature to

permit single stranded DNA to anneal to its primers. A stringent annealing temperature will increase the reaction specificity and best results with mycobacteria are achieved with annealing temperatures in the range of 55°C to 72°C. During the polymerisation step, a new strand of DNA is synthesised from the template DNA starting at the point where the primers anneal. The number of target DNA sequences doubles every time this series of reactions is repeated, so there is an exponential increase in copy number. The final step in this process is extension of the annealed DNA strands, depending on the length and concentration of the target sequence, at a temperature ranging from 70-72°C. The amplified DNA can then be analysed by agarose gel electrophoresis.

To avoid cross contamination that is a major problem in PCR, a set of micropipettes were devoted exclusively for DNA extraction and preparation of PCR reaction mixtures, at a designated bench. Post PCR products were handled with a different set of micropipettes at a different location, protective clothing was worn with frequent glove changes. Known positive and negative controls were included in all reactions after optimising each set of primers used initially.

In order to consider a positive result from a clinical material is genuine, not due to a contaminant from the laboratory, positive PCR amplicons had to fulfil the following criteria: (i) extraction controls and PCR negative controls were devoid of PCR products of the expected size; (ii) amplicons were consistently obtained from repeated amplifications from the same extracted template DNA and from further extracts of the same sample.

## **2.8.1 Procedures of DNA extraction from clinical specimens for PCR**

### **2.8.1.1 Processing of clinical samples for DNA extraction**

#### **(a) Paraffin embedded tissues**

Thin sections were cut from paraffin-embedded tissues with sterile disposable blades, and placed into 1.5 ml Eppendorf tubes. Tissues were deparaffinised by leaving them at 60°C for 15 minutes and then re-suspending in 500 µl xylene for a further 15 minutes at 60°C. Tubes were vortex mixed and centrifuged for 5 minutes at 13,000 g. Xylene was decanted and residual xylene removed by

adding 500  $\mu$ l of 70% and 100% ethanol, vortex mixing, and centrifuging at 13,000 g for 5 minutes respectively. The tissues were allowed to air dry and re-suspended in 50  $\mu$ l molecular biology grade water (Sigma).

*(b) Sputum samples*

Processed sputum samples kept at  $-20^{\circ}\text{C}$  after using for culture and staining were used for DNA extraction

*(C) Nasal swabs*

Per nasal calcium alginate swabs (Microbiological Supply Company) were used to collect nasal swabs. After collection, they were kept at  $4^{\circ}\text{C}$  and transported at room temperature to the study location. Each swab was cut above the calcium alginate part of the swab into a 2 ml microfuge tube containing 500  $\mu$ l molecular biology grade water and vortex mixed for 3 minutes. Swabs were pressed against the side of the tube to remove excess fluid and discarded. The suspension was used for DNA extraction.

#### **2.8.1.2 DNA extraction**

Three different DNA extraction methods for preparation of template DNA from samples were used as follows. Two negative controls, without sample were always processed as the sample tubes for all the extraction procedures.

##### **(1) DNA extraction using guanidine thiocyanate and silica suspension**

This method was based on the lysing and nuclease inactivating properties of guanidiumthiocyanate (GuSCN) together with the nucleic acid-binding properties of silica.

For DNA extraction, tissue samples were added using a sterile disposable plastic loop to 100  $\mu$ l of demineralisation solution in a screw capped Eppendorf with 1.5 to 2 mm glass beads (Merck). In the case of sputum and nasal swab samples, 100  $\mu$ l of the processed sample was added to 100  $\mu$ l demineralisation solution. Tubes were vortex mixed quickly and tubes with tissue samples were then placed on a Mini-Bead Beater® (Stratech Scientific Ltd) for 50 sec at maximum speed. Samples were allowed to disperse completely by incubating at  $56^{\circ}\text{C}$ ; sputum

samples overnight and tissues for 2-7 days, mixing tubes on the bead beater once every day. Two hundred and fifty micro litre (250  $\mu$ l) of lysis buffer L6 (Boom, *et al.*, 1990) was added to each tube and vortex mixed. Tubes were placed at 37°C in a water bath for about 2 hours to lyse the bacterial cells to release DNA. Tubes were vortex mixed and centrifuged for a minute, and the lysis buffer decanted into clean sterile tubes.

Twenty-five micro litres (25  $\mu$ l) silica suspension was added to each tube and placed on a Vortex Genie-2 mixer (Scientific Laboratory Supplies Ltd) at setting no.3 for an hour to allow the DNA to adsorb onto silica particles. At the end of the incubation, tubes were spun down for a minute and the silica supernatant collected into clean sterile tubes to process separately (to recover any DNA left behind in the supernatant). Washing buffer L2 (100  $\mu$ l) (Boom, *et al.*, 1990) was added to the silica deposit, vortex mixed and spun down for a minute at 13,000 rpm, and the supernatant was discarded. Pellets were washed twice with 200 $\mu$ l of 70% ethanol (-20°C) and once with acetone (-20°C) before draining the tubes and drying them in a heating block at 56°C. DNA was eluted in two stages from the dried pellet by adding 50  $\mu$ l molecular biology grade water each time and incubating at 65°C for an hour. The DNA extract was then aliquoted into small volumes and stored at -20°C until used for PCR.

**Processing of silica supernatant:** Supernatant was cooled on ice until completely chilled. Puregene Protein Precipitation Solution (Flowgen) (200 $\mu$ l) was added to each tube and tubes were vortex mixed vigorously for 20 seconds and spun down for 3 minutes. Supernatants (about 550 $\mu$ l) were added to tubes containing 600 $\mu$ l of isopropanol (-20°C) (Sigma) to precipitate the DNA. After mixing thoroughly (mixed by inversion 50 times) tubes were spun down for 3 minutes. Supernatants were discarded and any pellet was washed in 600 $\mu$ l 70% ethanol, drained and dried at 56°C. DNA was hydrated with 100 $\mu$ l molecular biology grade water at 65°C in a water bath for an hour or at room temperature overnight. Aliquots were stored at -20°C until use. (Donoghue, *et al.*, 2001).

## **(2) DNA extraction using Qiagen DNeasy® tissue kit (Qiagen)**

This method was employed to extract DNA from paraffin-embedded tissues. Tissue pellet was re-suspended in cell lysis buffer provided by the supplier and incubated with 20 µl Proteinase K (20mg/ml) at 56°C until the tissue had dissolved and the suspension became viscous. The Proteinase K was inactivated by heating to 70°C for 10 minutes in a water bath. Ethanol (200 µl) was added, and the mixture was applied onto a QIAamp spin column (Qiagen). This silica-gel spin column acts as a concentrator. After two rounds of washing, the DNA was eluted with 200 µl of the supplied buffer in two stages.

## **(3) DNA extraction using Pure Gene® extraction kit (Flowgen)**

This method was used to extract DNA from nasal swabs, which were carried out in category 3 laboratory. Puregene cell lysis solution (300 µl) and 2 µl of proteinase K (20 mg/ml) was added to 100 µl of processed sputa or nasal swab suspension. This was incubated at 56°C in a water bath for 24-48 hours. After incubation, tubes were pulse-centrifuged and further incubated at 37°C for 1 hour with 300 µl of L6 buffer, subsequently tubes were processed outside the category 3 laboratory.

Samples were cooled on ice and 100 µl of Puregene protein precipitation solution was added; the mixture was vortex mixed for 20 sec and centrifuged at 13 000 g for 3 minutes. The supernatant was carefully transferred to a fresh tube containing 300 µl of isopropanol (-20°C) and mixed well by inverting tubes at least 50 times. Tubes were incubated for 5 minutes at room temperature followed by centrifugation at 13 000 rpm for 1 minute to precipitate the DNA. The supernatant was discarded and the pellet washed twice in 70% ethanol (-20°C). The pellet was allowed to drain and air-dried. DNA was re-hydrated with Puregene rehydration solution (50 µl) at 65°C for 1 hour or at room temperature overnight.

## **2.8.2 Preparation of *M. tuberculosis* and *M. bovis* chromosomal DNA**

In category 3 laboratory DNA of MTB was prepared from a fresh culture of MTB by a boiling method. A colony of MTB was placed in 500 µl borate buffer saline pH 9.0, boiled for 15 minutes in a water bath. The suspension was vortex mixed and centrifuged at 13 000 g for 3 minutes. The supernatant was used as the DNA extract. Aliquots were stored at -20°C. Five micro litres (5 µl) of 1/10 dilution of this was used as a positive control in every PCR assay. *M. bovis* chromosomal DNA was also prepared using the same method described.

## **2.8.3 Selection of primers and PCR procedure**

Table 2.1 shows the primer sequence, size of PCR product and the PCR condition for each primer set used in this project. All the primers were from Oswel, Southampton, UK.

### **2.8.3.1 Primers common to all mycobacteria**

Primers that amplify a 439-bp fragment between positions 398 and 836 in the gene encoding the 65kDa protein were used to detect the presence of mycobacteria (Telenti, *et al.*, 1993). The 65kDa protein contains epitopes that are unique as well as common to various species of mycobacteria. Primers were used at a final concentration of 500nM.

### **2.8.3.2 Primers to detect the *Mycobacterium tuberculosis* complex**

The primers used in nested PCR to detect MTB were described by (Taylor, *et al.*, 1996). The target for DNA amplification was the repetitive element in the genome of the *M. tuberculosis* complex known as the insertion sequence IS6110. Copy numbers of IS6110 ranges from 0-25 in the genome of clinical isolates. The first pair of primers (P1 and P2); amplify a 123 bp region of IS6110 (Eisenach, *et al.*, 1991). The second set of primers (IS3 and IS4) bind internally with some overlap to P1 and P2, and amplify a 92 bp product. Both primer pairs were used at a final concentration of 500nM.

Another pair of primers used in this study to detect MTB DNA was NB3 and NB5. This primer set amplifies a 131 bp fragment from the gene encoding the 19-kDa protein, which is found in the MTB complex and *Mycobacterium scrofulaceum* (Mustafa, *et al.*, 1995). These primers were used at final concentration of 200nM.

#### **2.8.3.3 Primers for *Mycobacterium bovis***

Primers spanning the deletion region RD7 were originally described by (Sales, *et al.*, 2001). These primers were then modified in order to obtain smaller PCR products and nested PCR (BD1, BD2 and BD3, BD4), which increase the detection rate (Donoghue, H. D., Personal communication). These primers are specific for *M. bovis* and *M. bovis* BCG and absent in MTB.

#### **2.8.3.4 Primers for *Mycobacterium leprae***

Two pairs of primers (LP1, LP2 and LP7, LP8) were used for the specific amplification of *M. leprae* DNA (Donoghue, *et al.*, 2001). LP1 and LP2 primers are based on the repetitive sequence (RLEP), which is repeated 28 times in the *M. leprae* genome. LP7 and LP8 are based on the 18kDa antigen gene. Primers LP1, LP2 were used at a final concentration of 60 nM and LP7, LP8 at 200 nM.

#### **2.8.3.5 Primers for human DNA**

In order to test for any PCR inhibitors, PCR negative specimens were examined for mitochondrial human DNA. The specimen was considered inhibitory if no amplification product was observed. Primers RVM1 and RVM2, at a final concentration of 500nM of each primer, that amplify a 133 bp fragment (Barnes, *et al.*, 2000) were used to confirm MTB PCR negative results.

Target sequence	Primer sequence (5'-3')	Product size (bp)	PCR cycle condition	No of cycles
Hsp65	TB11: ACC AAC GAT GGT GTG TCC AT TB12: CTT GTC GAA CCG CAT ACC CT	439	*Touch down PCR then; 94°C 1 min, 60°C 1 min, 72°C 1 min	10 30
IS6110	P1: CCT GCG AGC GTA GGC GTC GG P2: CTC GTC CAG CGC CGC TTC GG  IS3: TTC GGA CCA CCA GCA CCT AA IS4: TCG GTG ACA AAG GCC ACG TA	123  92	94°C 1 min, 68°C 1 min 72°C 20 sec  94°C 1 min, 58°C 1 min 72°C 20 sec	25  25
19kDa protein	NB3: TCT TTC CGG ATG TTC AAG CA NB5: GTG ACG TTC TGG TCC TTA CC	131	*Touch down PCR then; 94°C 1 min, 58°C 1 min, 72°C 20 sec	12 30
RD7 deletion	BD1: ATC TTG CGG CCC AAT GAA TC BD2: CAA CGT CTT GCT GAC CGA CA  BD3: ATG AAT CGG CCG CGT TCG BD4: GAC CGA CAT CGG TGC CGC G	124  99	94°C 1 min, 60°C 1 min, 72°C 20 sec  94°C 1 min, 60°C 1 min, 72°C 20 sec	25  25
<i>M. leprae</i> RLEP	LP1: TGC ATG TCA TGG CCT TGA GG LP2: CAC CGA TAC CAG CGG CAG AA	129	*Touch down PCR then; 94°C 1 min, 59°C 1 min, 72°C 20 sec	10 30
<i>M. leprae</i> 18kDa antigen	LP7: TCA TAG ATG CCT AAT CGA CTG LP8: GGC ACA TCT GCG GCC AGC A	136	*Touch down PCR then; 94°C 1 min, 58°C 1 min, 72°C 20 sec	12 30
Mitochondrial human DNA	RV1: AGG GCC CGT ATT TAC CCT ATA G RV2: ATT TAG TTG GGG CAT TTC ACT G	133	94°C 40 sec, 52°C 1 min, 72°C 20 sec	40

**Table 2.1: Primer sequences and PCR procedures used in this study**

In all PCRs, there was an initial stage of strand separation at 94°C for 1-4 minutes, and final strand extension at 72°C for 10 minutes.\*Touch down PCR: Annealing temperature was increased to 70°C at the start of the reaction and reduced by 1°C/cycle to increase specificity

PCRs were performed individually and separately for MTB, *M. bovis* and human DNA. DNA extract (3 µl) was used in all PCRs in a final reaction mixture of 50 µl except for the nested PCR, where 1 µl of the stage one product was used. Promega master mix with final concentration of 25 units/ml of *Taq* DNA polymerase, supplied in a proprietary reaction buffer; pH 8.5, 200 µM dATP, 200 µM dGTP, 200 µM dCTP, 200 µM dTTP, 1.5 mM MgCl<sub>2</sub> was used to amplify DNA extracts from tissues. Pre-aliquoted double-strength PCR mix (Advanced Biotechnologies, Epsom) with a final composition of 75mM Tris-HCl (pH 8.8); 20mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 1.5mM MgCl<sub>2</sub>; Tween 20 0.01% v/v; 200 µM (each) dNTP; and 1.25 units *Taq* DNA polymerase was used to amplify DNA from sputum and nasal swabs. Bovine serum albumin (Sigma) was added to give a final concentration of 10mM as this has been shown to improve the yield (Forbes and Hicks, 1996). Amplification was carried out in a Phoneix thermal cycler (Helena Biosciences).

#### Touchdown PCR

Touch down PCR was performed to reduce the non-specific amplification, which is due to non-specific binding by one or both of the primers. This effect increases with the number of cycles and is much more likely in the early stages of the reaction when the template to product ratio is very low. Increasing the annealing temperature by 10°C at the start of the reaction increases the stringency of annealing and, thereby facilitates only specific primer template binding. As the amount of product increases the temperature is reduced with progressive cycles. This is because although a higher temperature gives greater accuracy it also produces a lower yield, and so by reducing the temperature the efficacy of the reaction is maximised.

Reaction mixtures containing extraction controls and molecular biology grade water instead of template DNA were used as negative controls. Reaction mixture containing MTB or *M. bovis* or human DNA was included with every set of tests as a positive control.

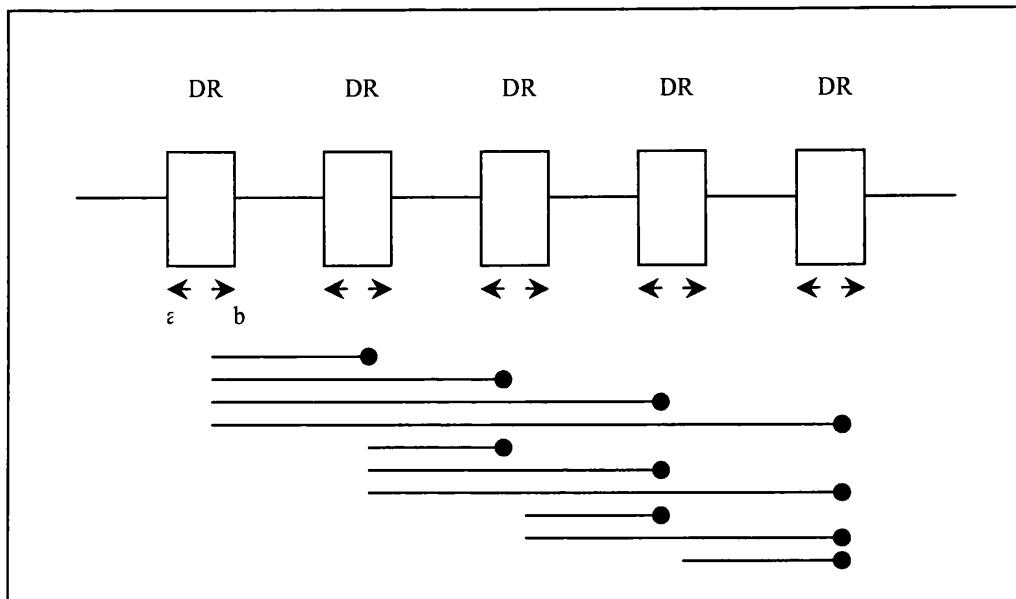
#### 2.8.4 Detection of amplified product

The amplified products were detected by direct analysis of 7 µl of PCR product, added to loading buffer (Sigma), on 3% Nusieve® agar (Flowgen) gels, which were electrophoresed at 8.8V/cm for one hour in TBE buffer, stained with ethidium bromide, and visualised by ultraviolet transillumination using a UVP camera and Labworks software (Ultra-Violet Products Ltd). The molecular size markers used were  $\varphi$ X174 *Hae* *III* digest (Promega), 123 bp ladder (Sigma) and 100 bp molecular ladder marker (Sigma).

### 2.9 Spoligotyping of DNA

Spoligotyping is currently a frequently used PCR typing method for the MTB complex. This method is based on the visualisation of the spacer DNA sequences between the 36-bp direct repeats (DRs) in the genomic DR region of the MTB complex (Kamerbeek, *et al.*, 1997). Each direct repeat is separated by a unique spacer sequence of 36 to 41-bp. One repeat sequence and the following spacer sequence together is termed a direct variable repeat (DVR). The number of DVRs varies from strain to strain, allowing this locus to be used as a genetic marker to differentiate strains. Two mechanisms have been proposed for the polymorphism at this locus: one is homologous recombination between adjacent or distant DVRs; the second is transposition of IS6110. The vast majority of the MTB strains contain one or more IS6110 elements in the DR region.

With this method, the presence or absence in the DR region of 43 spacers of known sequence can be detected by hybridisation of PCR-amplified spacer DNA (Fig 2.1) to a set of immobilised oligonucleotides, representing each of the unique spacer DNA sequences.



**Figure 2.1 Principle of the *in vitro* amplification of DNA within the DR region of MTB complex**

The use of two primers, DRa and DRb, for *in vitro* amplification, will lead to the amplification of any spacer or a stretch of neighbouring spacers and DR's

### 2.9.1 Preparation of DNA

DNA extraction was carried out using the same method described in section 2.7.2.

### 2.9.2 PCR analysis

Chromosomal DNA of MTB strains H37Rv and *M. bovis* BCG P3 were used as positive controls. Sterile distilled water was used as a negative control.

*Primers:* DRa: 5'-GGT TTT GGG TCT GAC GAC-3', biotinylated at 5' end

DRb: 5'-CCG AGA GGG GAC GGA AAC-3'.

*Reaction mixture:* A reaction volume of 25 µl Ready-To-Go™ PCR Beads (Amersham, UK) containing a final concentration of 1.5 units of *Taq* DNA

polymerase, 10mM Tris-HCl, 50mM KCl, 1.5 mM MgCl<sub>2</sub>, 200µM of each dNTP and stabilizer, including BSA was used for amplification. The volume of template DNA varied according to the concentration of DNA (typically 10 ng of purified chromosomal DNA is required to get a decent signal after hybridisation). Amplification was carried out in a DNA thermal cycler 480 (Applied Biosystems) and PCR was performed as follows: Reactions were initially denatured at 96°C for 3 min followed by 20-40 cycles of 96°C for 60 sec, 55°C for 60 sec, 72°C for 30 sec and a final extension at 72°C for 5 minutes.

### **2.9.3 Hybridisation with spacer-oligos**

Hybridisation of biotin-labelled PCR products to the immobilised spacer-oligos that represent spacers of known sequences was carried according to procedures described previously (Kamerbeek, *et al.*, 1997).

*Buffer preparation:* All buffers were pre-warmed before use. The following buffers were prepared from concentrated stocks, using demineralised water for dilution (quantities for one membrane).

250 ml 2x SSPE/0.1% (w/v) SDS, for use at 60°C

500 ml 2x SSPE/0.5% (w/v) SDS, for use at 60°C

500 ml 2x SSPE/0.5% (w/v) SDS, for use at 42°C

250 ml 2x SSPE for use at room temperature.

*Hybridisation:* 20 µl of the PCR product was added to 150 µl of 2x SSPE/0.1% (w/v) SDS. The diluted PCR product was heat-denatured for 10 min at 99°C and cooled on ice immediately. After the membrane (Pall Biosupport) was washed in 250 ml 2 x SSPE/0.1% (w/v) SDS at 60°C for 5 minutes, it was placed into the miniblitter (Immunetics) and the residual fluid was removed from the slots of the miniblitter by aspiration. The slots were carefully filled with diluted PCR products, and hybridised at 60°C for 60 minutes on a horizontal surface. The samples were removed from the miniblitter by aspiration and the membrane taken from the miniblitter using forceps. The membrane was washed twice in 250 ml 2xSSPE/0.5% (w/v) SDS at 60°C for 10 minutes.

#### **2.9.4 Chemiluminescent detection of spoligotype pattern**

The membrane was placed in a rolling bottle (Amersham, UK) and allowed to cool to prevent inactivation of the peroxidase in the next step in which 2.5 µl streptavidin-peroxidase conjugate (500U/ml) (Boehringer Mannheim) were added to 10 ml of 2 x SSPE/0.5% (w/v) SDS, and the membrane incubated at 42°C for 60 minutes. The membrane was washed twice in 250 ml of 2xSSPE/0.5% SDS for 10 minutes, rinsed twice with 250 ml of 2xSSPE for 5 min at room temperature. For chemiluminescent detection of hybridised DNA, the membrane was incubated for 1 min in 20 ml ECL® detection liquid (Amersham, UK.). The membrane was then placed between two transparent plastic sheets, in an X-ray exposure cassette in the dark room and an 8 x 10 inch X-ray film (Amersham, UK) inserted, followed by incubation at room temperature for 10 minutes. The film was developed using the developer and fixer (Sigma) each for 2 minutes. Spoligotype patterns were assessed visually. The signal of hybridisation in the test strains was compared with hybridisation signals of positive controls (H37Rv and BCG P3 DNA). Depending on the intensity of the signal, re-exposure of the membrane for shorter or longer periods was occasionally necessary. The film was allowed to drip-dry and the presence of spacers were visualised by exposure to film appearing as black squares.

#### **2.9.5 Analysis of spoligotype pattern**

Spoligotype patterns were analysed using Microsoft Excel.

#### **2.9.6 Regeneration of membrane**

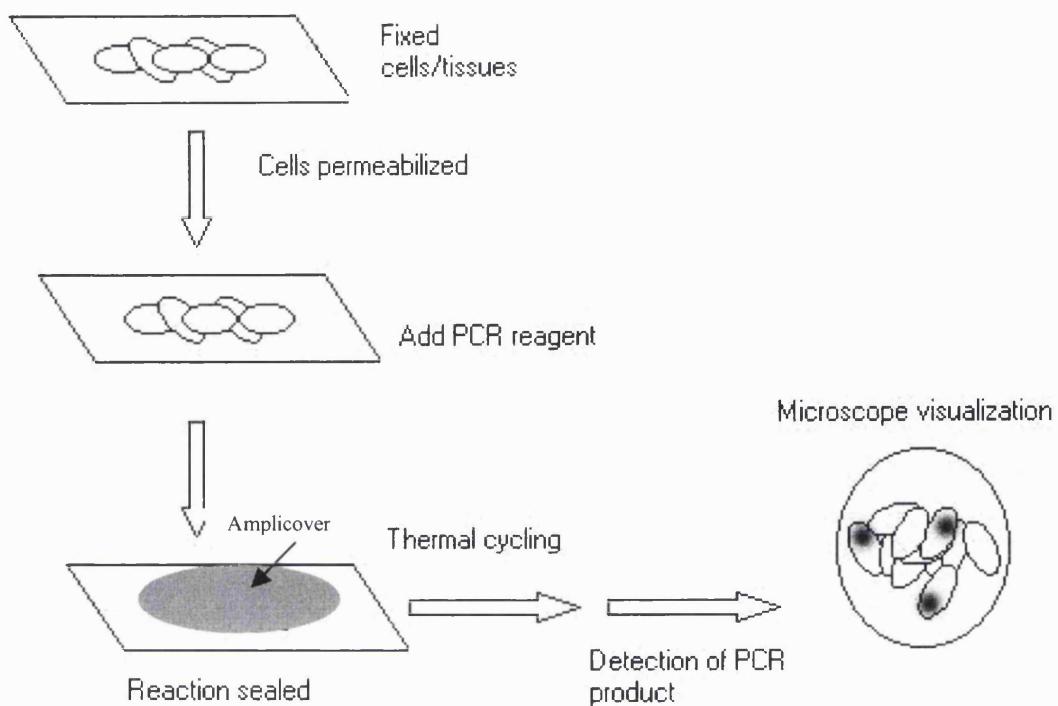
In order to regenerate the membrane for the next hybridisation, hybridised PCR products were disassociated from the membrane. The membrane was washed twice by incubation in 1% (w/v) SDS at 80°C for 30 minutes, followed by a wash in 20 mM EDTA at pH 8, for 15 min at room temperature. The membrane was sealed in a plastic bag to avoid dehydration and stored at 4°C until use.

To avoid contamination, preparation of the PCR reaction mixture was done in a separate room free of mycobacterial PCR products.

Spoligotyping experiments were carried out by the thesis author at the Research Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

## **2.10 In-situ PCR (ISPCR) for localization of MTB DNA in the paraffin-embedded lung tissues**

*In-situ* PCR is a technique that enables amplification of target sequences within intact cells (Fig 2.2). One of the earliest publications on *in situ* polymerase chain reaction (PCR) was by Haase (Haase, *et al.*, 1990). The technique was developed further by Nuovo, *et al.*, (1991) to localize different human papilloma viruses in formalin-fixed, paraffin-embedded tissue. However, *in-situ* amplification can be performed on virtually any tissue preparations, cell smears, cryostat sections, whole mounts, and cells in suspension.



**Figure 2.2: A graphical representation of the *in-situ* PCR process**

Several types of PCR amplifications (direct/indirect) are possible in non-disrupted cells and tissues. The term direct *in-situ* PCR describes the technique whereby a label is incorporated directly into the amplicon during the thermal cycling reaction and subsequently detected in order to localise the product at the site of amplification. The term indirect *in-situ* PCR describes an alternative technique whereby the amplicon is produced by thermal cycling without label incorporation. At the end of the cycling reaction, the amplified product is detected by standard *in-situ* hybridization using a labelled probe.

In this project, direct ISPCR was used. During ISPCR, a label is incorporated directly into the amplicon throughout the PCR process. Direct label incorporation results in the labelling of all nucleic acid synthesised during the PCR process. This results in high sensitivity with the detection of single copy genes being possible.

### **2.10.1 Preparation of sample**

Tissues were fixed in 4% Para formaldehyde in phosphate buffer and embedded in paraffin. Sections of 5  $\mu$ m were cut on silanized slides (Perkin Elmer) and incubated on a hot plate at 50-60°C for 36-48 hours to ensure maximum tissue adhesion on the slide. Tissues were first deparaffinized at 60°C and then in xylene for 12-18 hours at 60°C and followed by 30 min at 37°C. The sections were then immersed in fresh ethanol, followed by rehydration through a series of graded alcohols (starting with absolute, 75%, 50% and 25% alcohol kept at 4°C, 10 min in each). Finally, the slides were immersed in fresh distilled water prior to continuing with the pre-treatment, which is necessary in order to get penetration of the PCR components into the cells and to allow access of the target sequence for amplification.

### **2.10.2 *In-situ* PCR Pre-treatment**

Cells were rendered permeable by incubation with 0.02M HCl for 10 min and with 0.01% Triton X-100 (Sigma) for 90 sec in an attempt to partially solubilize the highly cross-linked histones in the nucleus, thus enabling the PCR components easier access to the target. Sections were then washed in PBS pH=7.3 and digested in pre-warmed 50 ml proteinase K (1 $\mu$ g/ml) for 30 min at 37°C to degrade many of the cross-linked proteins. Slides were boiled for 15-30 sec in a microwave to inactivate the proteinase K. Subsequently; hot slides were immersed in 20% acetic acid at 4°C for 15 seconds to reduce the level of endogenous alkaline phosphatase in the tissue. Finally, free DNA in the cells was fixed with 4% formaldehyde to prevent washing off. Slides were washed in PBS and air-dried.

### **2.10.3 *In-situ* PCR amplification**

PCR mixture (50 $\mu$ l) containing reaction buffer (2mM Tris-HCl, 10nM KCl pH 8.3), 3mM MgCl<sub>2</sub>, 200  $\mu$ M of dATP, dCTP, dGTP, dTTP with digoxigenin-11-dUTP mixture (Boehringer Mannheim) and 0.5 $\mu$ M of each MTB specific outer primers from IS6110 (P1, P2) was added to each section. Digoxigenin-dNTP was included in the reaction to anchor the product DNA *in situ* and prevent any

positive signal from diffusing into other cells. Subsequently, *Taq* DNA polymerase (Applied Biosystems) 12U in 0.6  $\mu$ l reaction mixture was added. Sections with the PCR mixture were sealed with an assembly tool (Applied Biosystems). Multiple samples were thermocycled by using a GeneAmp *in situ* PCR 1000 thermocycler (Applied Biosystems). The thermocycling profile included 35 cycles at 94°C for 1min, 70°C for 1min and 72°C for 1min for denaturation, annealing, and amplification steps respectively. Initial denaturation consisted of 94°C for 5 min. Amplification was completed with one cycle at 72°C for 10 min to complete DNA extension.

#### **2.10.4 Post *in-situ* PCR treatment**

Following thermocycling, the sample Amplicover disc (Applied Biosystems) was removed and slides were washed in Tris buffered solution (TBS) pH 7.6, to wash away all the remaining reaction components from the tissue section prior to detection. Specified amplified product was detected by the anti-digoxigenin alkaline phosphate system (Boehringer Mannheim) coupled with tetrazolium nitroblue and 5-bromo-4-chloro-3-indolyl phosphate toludine salt (BCIP). Tissues were incubated for 30 min with 1:500 anti-digoxigenin antibody (Fab fragments) (Boehringer Mannheim) with 2% foetal serum (Sigma) as a blocking agent. Slides were washed in TBS and then incubated with the substrate 1:50 dilution and kept in the dark but monitored at intervals until colour development (purple precipitate) was satisfactory and negative controls remained colourless. Colour development was 10-30 min for a satisfactory signal without appearance of background. At this point, the reaction was stopped by immersion of slides in TSB. Tissues were counterstained with nuclear fast red (Dako) and dehydrated before being permanently mounted.

Negative controls included non-infected mouse lung, and lung autopsies from patients identified as negative for MTB DNA in the preliminary experiments. An extra section of each sample was included on the same slide as the test section to which PCR mix without *Taq* polymerase was added, to avoid false positive due to DNA repair.

## **Chapter 3**

### **3. Multiple skin testing in school children from Northern and Southern Sri Lanka**

#### **3.1 Introduction**

Early immunisation is required to protect children from dangerous diseases such as tuberculosis. Sri Lanka is one of the countries where BCG is given at birth or soon thereafter. Administration of early immunisation within the first weeks of life is critical, especially in areas with high prevalence of HIV-infection, as vaccination with live BCG can cause delayed or disseminated BCG infection in HIV-infected infants (Besnard, *et al.*, 1993, Marks, *et al.*, 1993, Grange, 1998). Therefore, the prognosis of HIV infection must be taken into account when vaccination is being considered. On the other hand, the chances of infants getting exposed to TB infection in high TB endemic areas during their first weeks of life is high, and in places like Sri Lanka where HIV infection is rare, early immunisation is indicated.

T-cell immaturity in infants has not been considered in immunisation schedules with BCG. However, a study on proliferative lymphocyte responses and cytokine production to purified protein derivative (PPD) demonstrated development of a Th-1 type response in children vaccinated with BCG at birth and at the age of 2 and 4 months (Marchant, *et al.*, 1999).

There is consistent evidence that tuberculin reactivity after BCG vaccination in infancy wanes rapidly and this is primarily affected by age at vaccination. If the vaccine is given in infancy, tuberculin reactivity wanes rapidly in all individuals (Karalliedde, *et al.*, 1987). Waning of tuberculin response was observed by age of five in Sri Lankan school children who received the vaccine at birth. On the other hand, BCG vaccination administered after the first year of life resulted in persistent tuberculin reactions in a substantial proportion of vaccine recipients. A significant proportion of Danish school children, vaccinated at age 6 years retained tuberculin reactivity five years after vaccination (Horwitz and Bunch-Christensen, 1972).

Nevertheless, early immunisation is required in areas where environmental mycobacteria are common. Using guinea pigs, Palmer and Long found that BCG could increase the partial degree of immunity induced by prior contact with an environmental mycobacterium (Palmer and Long, 1966). Contact with environmental mycobacteria primes the immune system, and the type of response evoked to tuberculin skin test reagent in an individual depends upon the species of mycobacteria present in the environment and the frequency with which they are met. There are two patterns of cellular response to mycobacteria. The 'Listeria' type (non-necrotising), protective type; or the 'Koch type' (necrotising), less protective or which reduces the level of protective immunity (Stanford, *et al.*, 1981). Mycobacteria differ in their ability to induce these two patterns of reactions. While *Mycobacterium nonchromogenicum*, *M. vaccae* and *M. leprae* induce the Listeria-type response; *M. kansasii*, *M. scrofulaceum* and MTB are capable of inducing either response (Stanford, *et al.*, 1981). BCG vaccination can induce and boost the type of pre-existing response. Therefore, BCG affords protection when it elicits or boosts Listeria-type reactivity but is ineffective or even harmful when it boosts the Koch-type reactivity (Rook, *et al.*, 1981, Stanford, *et al.*, 1981). The latter, negative effect of BCG, accounts for the Chingleput findings, due to the high prevalence of the Koch-type of reactivity amongst those vaccinated (Stanford, 1983).

A study done in Agra, India also demonstrated the effect of high prevalence of environmental mycobacteria on the efficacy of BCG vaccination and reaction to PPD. When children vaccinated after the age of three in Agra were tested for tuberculin reactivity two years after vaccination, a Koch-type response was observed in a significant proportion of children (Stanford, *et al.*, 1987a). On the other hand, a tuberculin skin test carried out 2 years after vaccination on school children in another Indian town, Ahmednagar, revealed an almost identical reaction size that was observed 10 years after BCG administration in the UK, where BCG is considered very protective (Stanford, *et al.*, 1987b). In Agra, a very high level of sensitisation was found to most of the antigens prepared from environmental mycobacteria (new tuberculins), but a preliminary skin test survey in Ahmednagar showed that the rate of sensitisation with age was much lower

than in Agra, indicating the influence of exposure to environmental mycobacteria on the protective capacity of BCG vaccine.

Exposure to environmental mycobacteria can give protection against TB depending on the type of mycobacteria to which an individual is sensitised. Individuals with evidence of exposure to 'fast growers' are at reduced risk of contracting both TB and leprosy, compared to individuals who are sensitised to 'slow growers' such as *Mycobacterium scrofulaceum*, *M. intracellulare* and *M. fortuitum* (Fine, *et al.*, 2001). The evidences of impact of exposure to environmental mycobacteria on the susceptibility to TB may explain geographic distribution of mycobacterial diseases.

Pinto and colleagues studied the sensitisation of Sri Lankan rural populations to various new tuberculins (Pinto, *et al.*, 1972). They found that sensitisation was higher in the plains at sea level, while the percentage of reactors decreased with increase of altitude. The present work has used skin test data to study the prevalence of mycobacterial species in two sea level geographical locations in the Northern and Southern parts of Sri Lanka.

When deciding the age of vaccination, the prevalence of other infectious diseases also needs to be considered, not only the prevalence of environmental mycobacteria. It is now well established that a Th-1 type response is required for protection against TB. Significant level of production of IL-4 to PPD has been demonstrated in 2-4 months old unvaccinated children in Gambia, suggesting the establishment of type 2 response as early as 2 months after birth (Marchant, *et al.*, 1999). As BCG boosts the pre-existing type of response, early immunisation is preferable in these locations.

Tuberculin skin tests are used to ascertain active or latent infection, and for testing priming of the immune response after BCG vaccination. There are no methods available to distinguish tuberculin reactions caused by BCG vaccination from those caused by natural MTB infection or by environmental mycobacteria (Snider, 1982). Studies have shown post-vaccination-induced tuberculin reactivity

to range from no induration to reactions of 19 mm (Horwitz and Bunch-Christensen, 1972, Karalliedde, *et al.*, 1987).

Nevertheless, the general belief is that post-vaccine tuberculin conversion is evidence of successful BCG vaccination. Based on this belief, tuberculin-negative subjects are repeatedly vaccinated in many countries. In Sri Lanka, a second BCG vaccine was previously given at the age of 10 years without prior tuberculin testing through the medical service. However, this programme has been discontinued since 1987. Karalliedde and colleagues (1987) found a significant increase in the tuberculin response 3 months after a second BCG vaccination in Sri Lankan children. A similar situation was recorded in Kuwait (Shaaban, *et al.*, 1990). However, no scientific study has shown whether BCG re-vaccination increases immunity and retains the trend of tuberculin positivity over time after the second BCG vaccination.

### **Aims of the study**

The purpose of this investigation is to study:  
the effect of geographical variation and ethnic origin on the sensitisation of school children to tuberculin and new tuberculins; and  
the impact of re-vaccination on the sensitisation to tuberculin and new tuberculins over a period of 5 years.

### **3.2 Materials and methods**

Data in this study were collected during 1987 and provided by Prof. John Stanford for analysis.

#### **3.2.1 Skin test antigens**

Altogether 8 skin test reagents, including tuberculin, prepared in London from different mycobacterial species, were used. These antigens and their origin are listed in Table 3.1.

Skin test antigens	Abbreviation	Species of origin	Children tested
Tuberculin <sup>a</sup>	T	<i>M. tuberculosis</i>	1099
Avumin	A	<i>M. avium</i>	512
Vaccin	V	<i>M. vaccae</i>	587
Scrofulin	S	<i>M. scrofulaceum</i>	587
Marinin	M	<i>M. marinum</i>	512
Nonchromogenecin	N	<i>M. nonchromogenicum</i>	512
Burulin	B	<i>M. ulcerans</i>	444
Leprosin	L	<i>M. leprae</i>	143

**Table 3.1: The skin test antigens used, their origin and number of children tested with each individual antigen**

<sup>a</sup> The tuberculin used in this study was a sonicate of *M. tuberculosis*, not PPD

The preparation of these skin test antigens has been described elsewhere (Stanford, *et al.*, 1975). Briefly, cultures of mycobacteria were ultrasonicated to disintegrate the bacilli and release proteins, centrifuged, and filter-sterilised. The protein concentrations used were 2 µg/ml for all reagents except vaccin and nonchromogenecin (both 20 µg/ml) and leprosin (10 µg/ml). Three sets each contain four skin test reagents, one of which was tuberculin, were used. Each child tested received four reagents: two intradermal injections of 0.1 ml of two skin test reagents were administered on the volar aspect of each forearm. The set of skin test antigens used, the number of children tested and their BCG status are tabulated in Table 3.2. Tuberculin was always administered on the left proximal forearm. Responses were read after 72 hours. A diameter of induration of 2 mm or more was taken as a positive response.

BCG status	Set of skin test antigens used			
	T, A, M, N	T, S, V, L	T, S, V, B	Total
Positive	422	124	380	926
Negative	66	8	37	111
Re-vaccinated	24	11	27	62
Total	512	143	444	1099

**Table 3.2: Total number of children tested with different set of antigens grouped according to BCG status**

### 3.2.2 Children tested

Multiple skin testing was carried out on children attending schools in two districts in Sri Lanka. These were two village schools in the Jaffna district and three village schools in the Hambanthota district.

Both Jaffna and Hambanthota are sea level areas. Puttur and Urumbrai are small rural areas 5 miles away from Jaffna town, the third biggest town in Sri Lanka. Jaffna is situated in the northern part of Sri Lanka and inhabited by Tamils. The climate is intermediate in-between wet and dry. The main income source of rural population of Jaffna is farming.

Area	School	Total children tested	6-10 years		11-15 years		
			BCG	No BCG	BCG	No BCG	Re-vaccinated
Jaffna	Urumbrai (Tamils)	361	182	27	144	8	NT
	Puttur (Tamils)	260	104	25	131	NT	NT
Hambanthota	Hambanthota (Singhalese)	218	114	11	85	4	4
	Dharmakabeer (Muslims)	226	88	25	68	12	33
	St Mary's (Singhalese & Muslims)	34	NT	NT	9	NT	25

**Table 3.3: Number of children tested at different schools grouped according to age, ethnicity and BCG status.**  
 NT-Not Tested

Hambanthota is on the south coast of Sri Lanka, with a dry climate, and situated at a distance of 240 miles from Jaffna. Fishing is the main occupation of this rural population. The main ethnic groups in this area are Singhalese and Muslims who are of Arab origin.

The number of children tested at different schools according to their age, ethnicity, and BCG status is given in Table 3.3. Children were arbitrarily divided into two age groups of 6-10 years and 11-15 years. Previous BCG vaccination was recorded for each child based on the presence of a post-vaccination scar.

### **3.2.3 Statistical analysis**

SPSS 9.05 for Windows software was used to perform the statistical analysis of the data. The chi-square test was used to compare the percentage of reactors of different groups. A nonparametric test, Mann-Whitney, was used to compare means of reaction sizes. A p-value of  $<0.05$  was considered statistically significant, and 95% confidence intervals were calculated for the main results.

## **3.3 Results**

### **3.3.1 Sensitivity to tuberculins in the whole series**

Taken all together, 85% of the children tested had been BCG vaccinated and 41% and 16% of vaccinated and unvaccinated respectively reacted to tuberculin. A complete picture of reactivity to all skin test antigens grouped according to age and BCG status is given in Table 3.4. Cumulative data shows highest number of positive reactors to avumin amongst both vaccinated and unvaccinated children and lowest to Marinin. The mean indurations to all skin test reagents are given in Table 3.5.

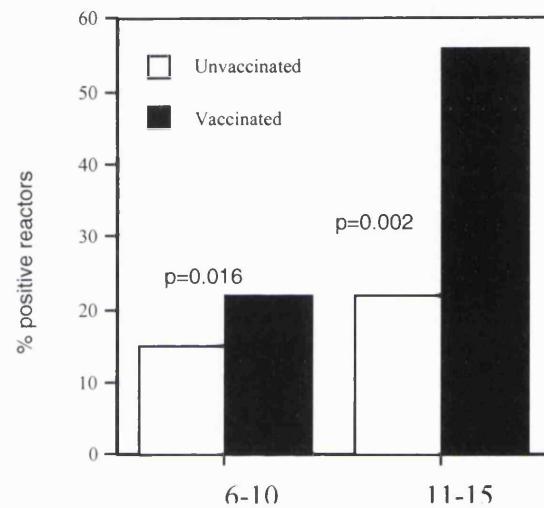
Skin test antigen	All ages			6-10 years		11-15 years		
	Immunised	Non-immunised	Re-immunised	Immunised	Non-immunised	Immunised	Non-immunised	Re-immunised
Tuberculin	377/926 (41%)	18/111 (16%)	36/62 (58%)	131/488 (27%)	13/88 (15%)	246/43 (56%)	5/23 (22%)	36/62 (58%)
Auramin	260/422 (62%)	36/66 (55%)	20/24 (83%)	112/220 (51%)	27/51 (53%)	148/202 (73%)	9/15 (60%)	20/24 (83%)
Vaccin	196/504 (39%)	11/45 (24%)	20/38 (53%)	92/268 (34%)	8/37 (22%)	104/236 (44%)	3/8 (38%)	20/38 (53%)
Scrofulin	238/504 (47%)	12/45 (27%)	17/38 (45%)	99/268 (37%)	10/37 (27%)	139/236 (59%)	2/8 (25%)	17/38 (45%)
Marinin	159/422 (38%)	11/66 (17%)	15/24 (63%)	52/220 (24%)	8/51 (16%)	107/202 (53%)	3/15 (20%)	15/24 (63%)
Nonchromogenicin	214/422 (51%)	39/66 (59%)	19/24 (79%)	86/220 (39%)	23/51 (45%)	128/202 (63%)	7/15 (47%)	19/24 (79%)
Borulin	159/380 (42%)	9/37 (24%)	16/27 (59%)	87/268 (32%)	9/37 (24%)	72/111 (65%)	NT	16/27 (59%)
Leprosin	81/124 (65%)	3/8 (38%)	8/11 (73%)	NT	NT	81/124 (65%)	3/8 (38%)	8/11 (73%)

**Table 3.4: Data collected from all five schools showing the effect of BCG vaccination in association with age on the positivity to each of the skin test reagents.**

Skin test antigen	BCG Vaccinated (mm)	BCG Unvaccinated (mm)
T	9.70	9.10
A	8.36	7.59
V	7.64	5.75
S	8.06	7.08
M	8.02	7.50
N	6.44	4.03
B	7.21	6.38
L	8.95	4.00

**Table 3.5: The average of the mean diameters of indurations of positive reactors**

As expected, within the BCG-vaccinated population, increasing age was associated with increasing positivity to tuberculin and new tuberculins. However, there was no significant difference in the reactivity to skin test reagents amongst unvaccinated 6-10 and 11-15 year olds. BCG vaccination significantly influenced the reactivity to tuberculin amongst both age groups (Fig 3.1). In contrast to this, BCG vaccination had no effect on the reaction to new tuberculins amongst the younger age group. Nevertheless, BCG vaccination significantly increased the reactivity to slow growers, marinin and scrofulin in the older age group (Fig 3.2). Increase in age also considerably increased the sensitivity to slow growers.



**Figure 3.1: Histograms showing proportion of positive reactors to tuberculin amongst 6-10 and 11-15 year olds according to their BCG status**

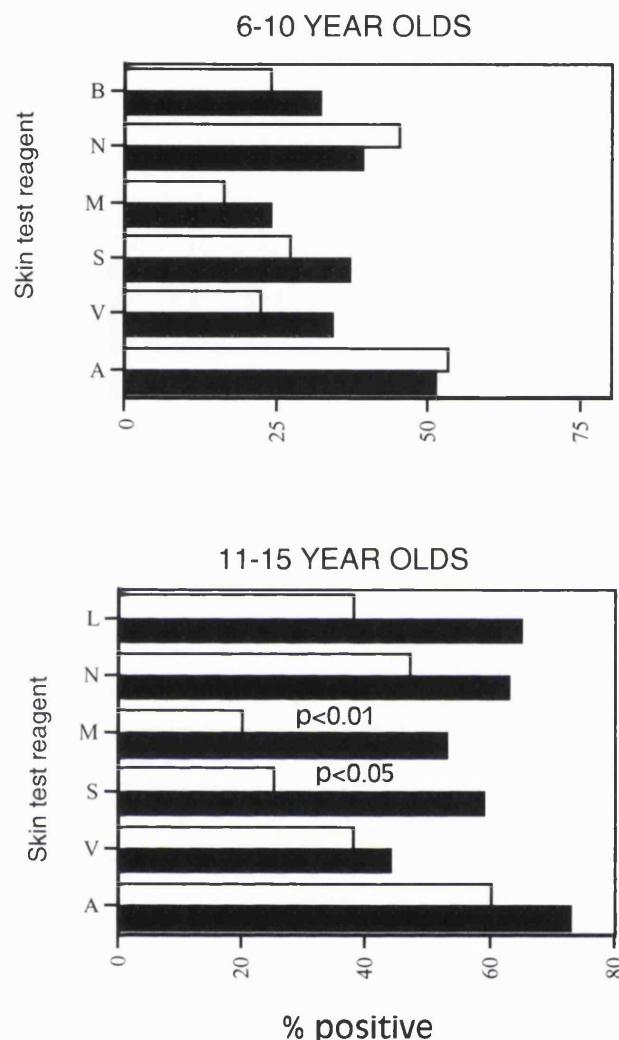
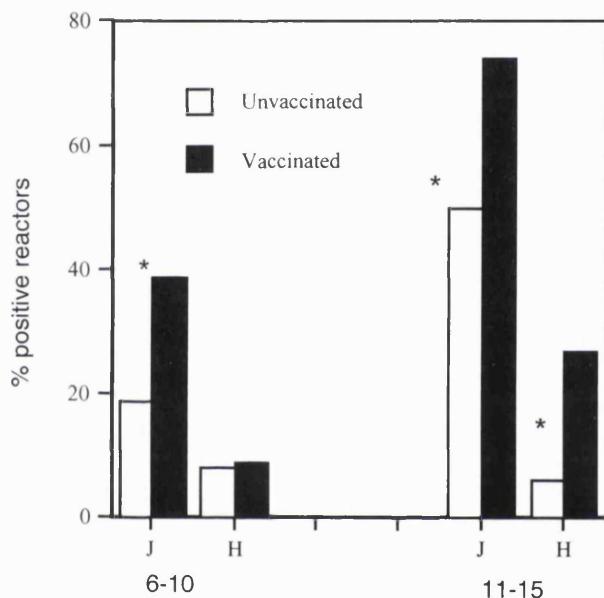


Figure 3.2: The association between BCG vaccination and the positivity to new tuberculins in different age groups

Clearblocks represent positive reactors among unvaccinated children and black blocks represent positive reactors among vaccinated children

### 3.3.2 Skin sensitivity to tuberculin in different geographical areas

Clear-cut variation was observed in the reactivity to tuberculin between the two geographical areas. Compared to Jaffna, the proportion of positive reactors to tuberculin was very low amongst vaccinated children living in Hambanthota (Fig 3.3). Positive reactors to tuberculin amongst unvaccinated children were 23% and 8% in Jaffna and Hambanthota respectively.



**Figure 3.3: The variation in reactivity to tuberculin in Jaffna (J) and Hambanthota (H) in association with BCG status and age**

\* significant difference <0.05

The overall distribution of reaction size to tuberculin in the schools tested is shown in figure 3.4 & 3.5. The distribution of reaction size was unimodal amongst the younger age group in all the schools. With increasing age there was a shift from an apparently unimodal to an apparently bimodal pattern in the Jaffna schools, but the distribution remained unimodal in Hambanthota schools. Significant increase in the reaction size was observed in the both age group of children from Jaffna compared to children from Hambanthota district.

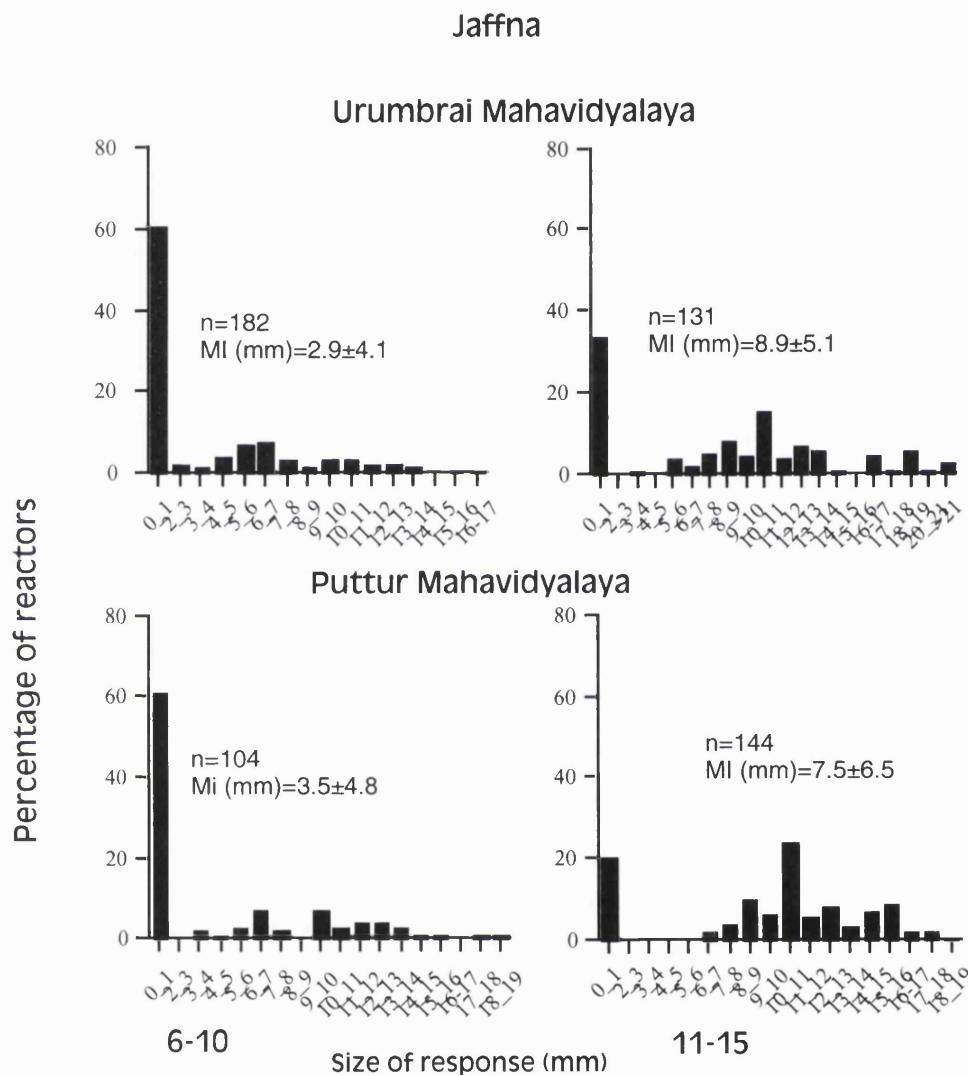


Figure 3.4: Frequency of size of response to tuberculin in all vaccinated children tested at individual school in Jaffna  
MI: Mean induration

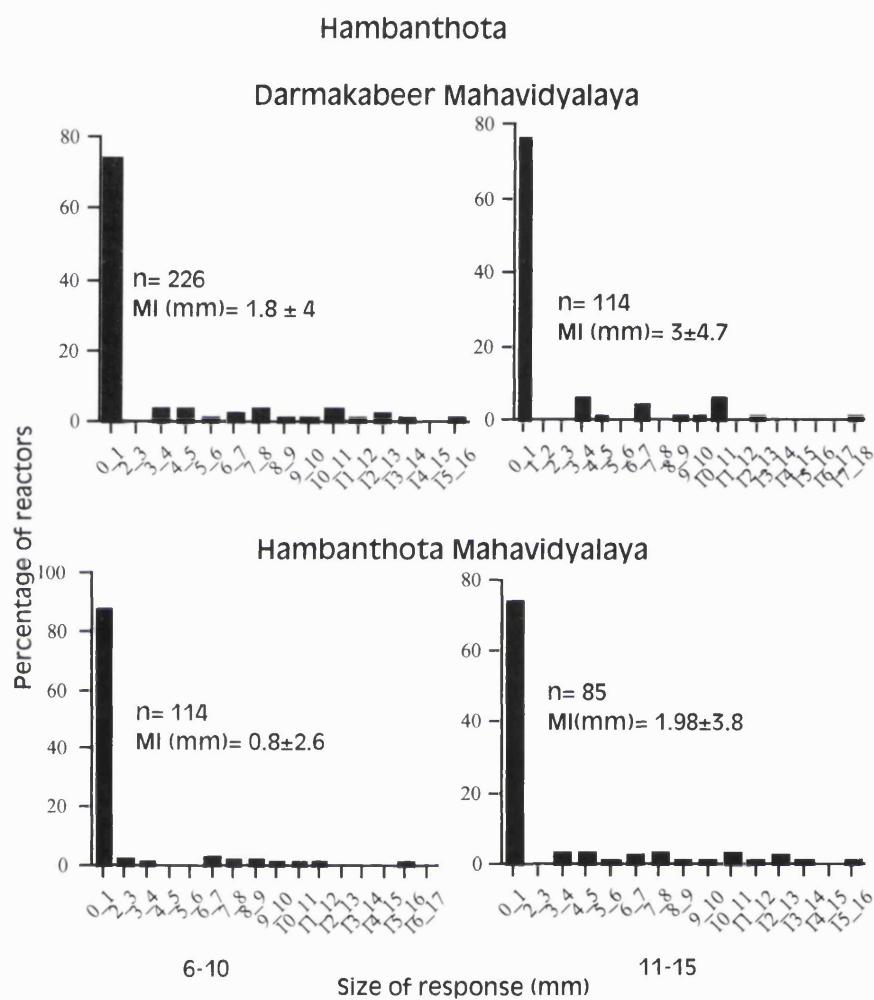


Figure 3.5: Frequency of size of response to tuberculin in all vaccinated children tested at individual schools in Hambanthota  
MI: Mean induration

Percentage of positive reactors showing reaction size of 10 mm or more to tuberculin and aviumin amongst vaccinated children at individual schools is given in table 3.6.

School	Tuberculin		Aviumin	
	6-10	11-15	6-10	11-15
Urumbrai	28.7%	45.1%	6.2%	28.7%
Puttur	24.1%	64.1%	22.5%	72.5%
Hambanthota	3.6%	10.7%	7.2%	13.6%
Darmakabeer	4.7%	10.0%	21.1%	44.1%

**Table 3.6: Table showing the percentage of reactors elicited 10 mm or more reaction size to tuberculin at individual school**

Variation between the schools in Jaffna in skin test reactivity to tuberculin was observed amongst older children. These schools are only few miles away from each other.

### 3.3.3 Skin test reactivity to new tuberculins

Variation in positivity to each skin test reagent amongst children from four schools is shown in figure 3.6 & 3.7. The pooled skin test data for all the five schools is given in Table 3.7.

	6-10				11-15				
	BCG		No-BCG		BCG		No-BCG		Re-vaccinated
<b>Tuberculin (+)</b>									
Urumbrai	72/183	39%	8/27	30%	96/144	67%	4/8	50%	NT
Puttur	41/104	39%	2/25	8%	105/131	80%	NT	NT	
Hambanthota	13/114	11%	0/11	0%	22/85	26%	0/3	0%	0/4 0%
Dharmakabeer	6/87	7%	3/25	12%	16/69	24%	1/12	8%	21/33 64%
St Mary's	NT		NT		6/9	66%	NT		14/25 60%
<b>Fast growers (+)</b>									
Urumbrai	68/183	37%	12/27	44%	82/144	61%	5/8	63%	NT
Puttur	46/104	44%	9/25	36%	105/131	80%	NT	NT	
Hambanthota	38/114	32%	3/11	36%	25/85	29%	1/3	33%	3/4 75%
Dharmakabeer	19/87	22%	9/25	36%	30/69	43%	6/12	50%	22/33 66%
St Mary's	NT		NT		1/9	11%	NT		12/25 48%
<b>Slow grower (+)</b>									
Urumbrai	103/183	56%	16/27	59%	101/144	70%	5/8	63%	NT
Puttur	61/104	59%	12/25	48%	110/131	83%	NT	NT	
Hambanthota	34/114	30%	2/11	18%	36/85	42%	1/3	33%	2/4 50%
Dharmakabeer	17/87	20%	6/25	24%	29/69	42%	6/12	50%	25/33 76%
St Mary's	NT		NT		5/9	56%	NT		10/25 40%

**Table 3.7: Table showing the affects of age and BCG vaccination in each school for tuberculin, pooled data for reagents prepared from fast growers and pooled data for reagents from slow growers.**

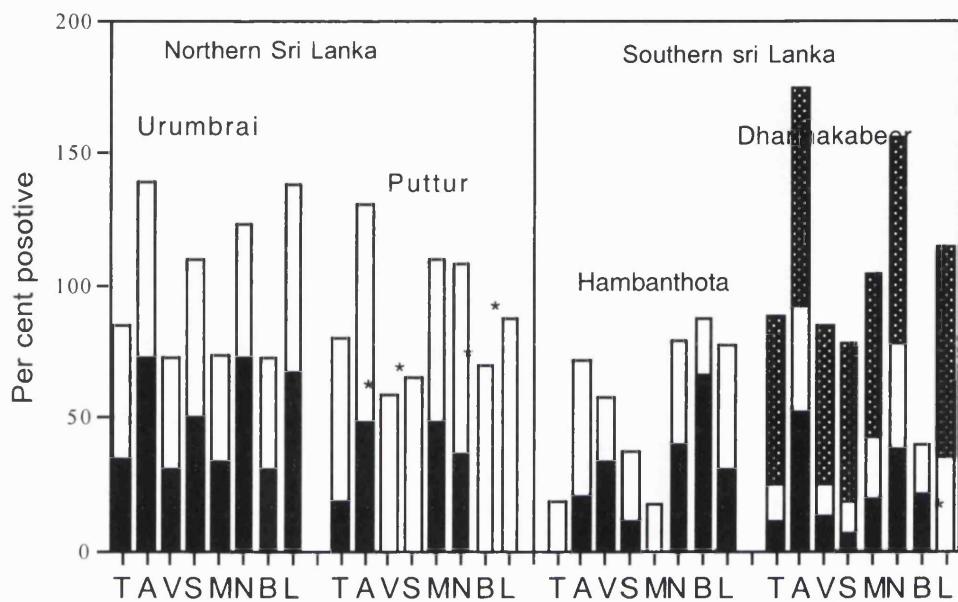


Figure 3.6: Stack columns showing the percentage of positivity at the individual schools to eight skin test reagents

Black blocks, clear blocks and blocks with dots represent percentage of positivity amongst unvaccinated, vaccinated, and re-vaccinated children respectively. \*:V,S,B, and L were not tested on unvaccinated children in Puttur; \*:L was not tested on unvaccinated children in Darmakabeer

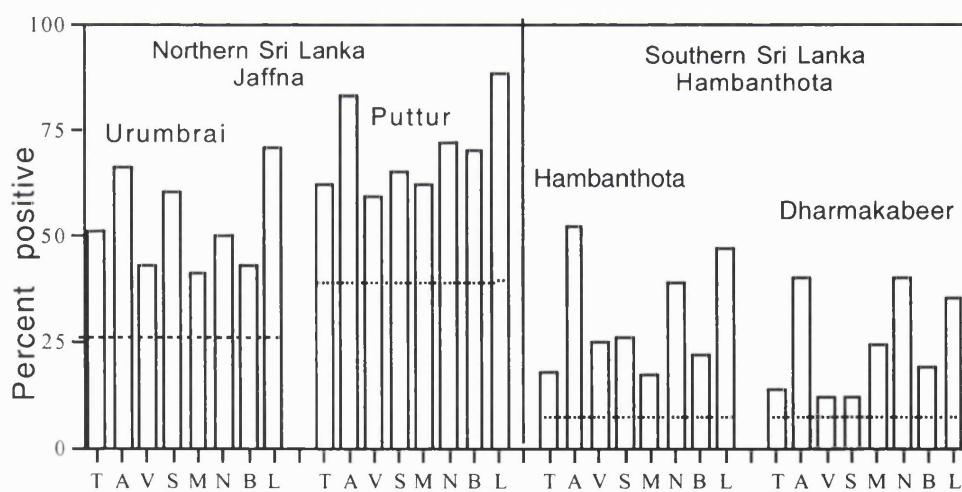


Figure 3.7: Bar graph showing the percent positive amongst vaccinated children at the individual schools to eight skin test reagents. The broken lines indicate the level below which reactions are to common antigens (group (i)).

### **3.3.4 Tuberculin reactivity in re-vaccinated children**

Tuberculin reactivity was assessed in re-vaccinated children 1-2 years and 4-5 years after the second vaccination. Reactivity increased significantly 1-2 years after the second vaccination, but the reactivity did not increase after that in association with increasing age (Fig 3.8). The reaction size also significantly increased 1-2 years after second vaccination and remained at the same level even 4-5 years after the second vaccination (Fig 3.9). A significant increase in the reactivity to other skin test antigens, except scrofulin, was found after the second vaccination (Fig 3.10). Conclusions about any affect of re-vaccination on the recognition of environmental mycobacteria in association with increasing age cannot be drawn as there were very few re-vaccinated >14 year olds in the study population.

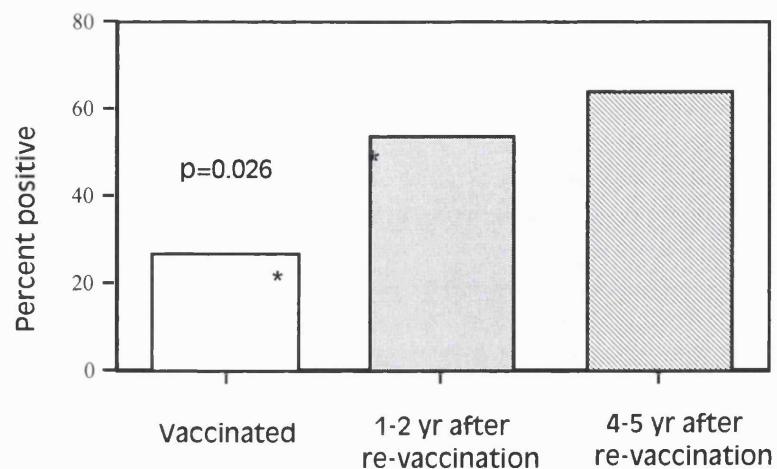


Figure 3.8: Percentage of positive reactors to tuberculin amongst vaccinated and re-vaccinated children in association with age

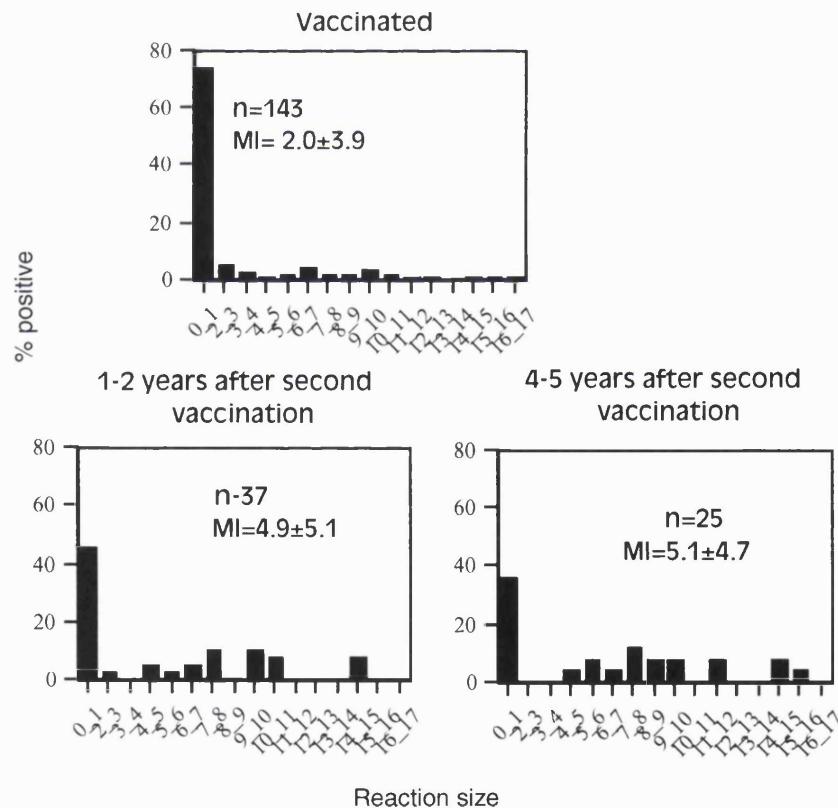
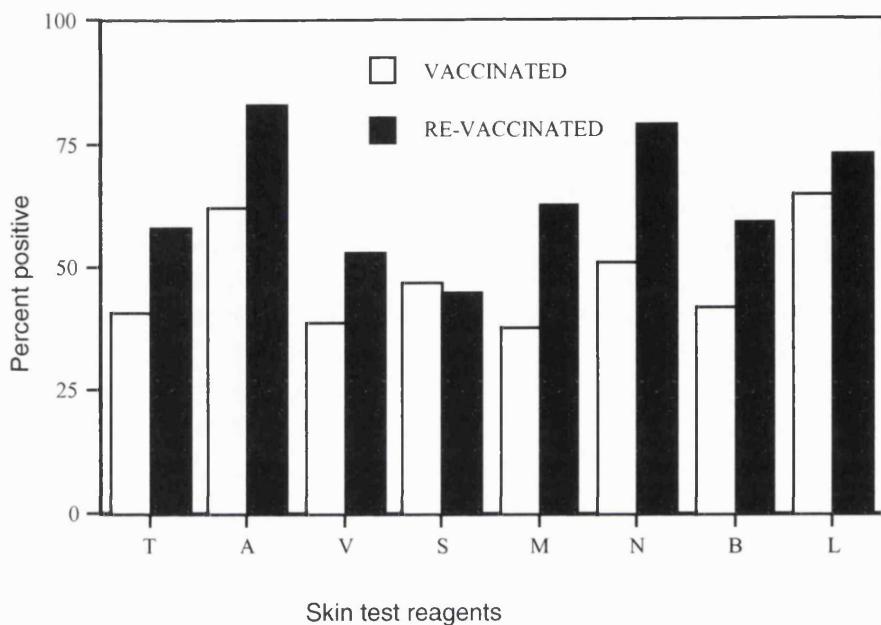


Figure 3.9: Distribution of reaction size in vaccinated and re-vaccinated children



**Figure 3.10: Graph showing the percentage of positive responders to each of the skin test antigens for children grouped accordingly to their BCG status.**

### 3.4 Discussion

Sri Lanka is a small island situated southeast of the southern-most tip of India. There are only few published data available on the mycobacteria of the Sri Lanka habitat as well as on the prevalence of non-tuberculous mycobacterial infections. None of this published work studied the skin test patterns of the Jaffna Tamil population in this country. A small re-vaccinated population included in this present study enabled the effect of revaccination in protective immunity to be investigated. Furthermore, investigation of reaction pattern of skin test in two different ethnic groups living in the same area enabled a study of the effect of genetic variation on skin test reaction.

Four categories of antigens have been demonstrated in mycobacteria by immunodiffusion analysis: group (i) antigens: common to all mycobacteria; group

(ii) antigens: found in slow growers; group (iii) antigens: found in fast growers; and group (iv) antigens: limited to strains of individual species (Stanford and Grange, 1974). In this study, antigens prepared from ultrasonicated *M. tuberculosis*, and other environmental mycobacterial cultures were used. These extracts have been shown to be rich in more species-specific antigens and relatively poor in group (i) antigens (Stanford, *et al.*, 1975, Magnusson, 1986). Thus, skin test reagents prepared from these extracts have shown greater specificity for delayed hypersensitivity to species-specific antigens compared to PPD, which show some specificity and much cross reactivity (Paul, *et al.*, 1975).

Increased species-specific skin test positivity is observed in vaccinated people in areas where BCG vaccination is effective. In other words, recognition of common antigens primed by BCG vaccination facilitates recognition of species-specific ones (Stanford, 1991a). Therefore, the enhanced responsiveness to tuberculin and to new tuberculins in vaccinated Sri Lankan school children compared to unvaccinated children, strongly suggests a valuable role for BCG in protection against tuberculosis. However, the observation of large reaction size to tuberculin amongst older children in Jaffna questions the efficacy of BCG in this area (Fig 3.4). A small reaction size to tuberculin was observed amongst younger children attending schools in the two districts included in this study. However, a significant increase in the reaction size was observed amongst older children attending schools in the Jaffna district. Large reaction size has shown to be associated with lack of protective immunity (Th2 response) after vaccination (Tuberculosis prevention trial 1979). Frequent contact with potentially pathogenic environmental slow-growing species such as *M. scrofulaceum* (Burma) or *M. intracellulare* (South India) can induce a Koch response to their own group (iv) antigens without the development of clinically apparent disease (Stanford, 1991b), which can increase the susceptibility to tuberculosis. In Jaffna, slow-growers are universally present and are frequently met by children, compared to children in Hambanthota district (Fig 3.7). The Madras BCG trial revealed a high incidence of TB amongst those showing strong reaction to avumin. Therefore, the strong response to tuberculin and avumin amongst older children in Jaffna district is worrying.

Human response to mycobacterial antigens can be divided into three categories: individuals reacting to common antigens - category 1 (reacting to all the set of skin test antigens); individuals reacting to none - category 2 (anergy); and individuals reacting to some but not all - category 3 (reacting to species-specific antigens) (Lockwood, *et al.*, 1987). Studies on adults have shown that up to 20% of individuals in a highly sensitised populations will respond to all new tuberculins, even to those prepared from species absent in that environment. In this study, burulin prepared from *M.ulcerans* was used to establish the level of responsiveness due to cross reactivity. This species has never been reported as a cause of disease in Sri Lanka until recently, therefore it is reasonable to assume it is absent in Sri Lanka (Stanford, J. L. personal communication). Therefore, any response to burulin, is assumed be to shared antigen rather than to species-specific antigens' i.e. due to category 1 reactors in that population. In Urumbrai, Puttur, Hambanthota, and Darmakabeer, 25%, 48%, 6%, and 6% respectively of category 1 responders were found. Therefore, any positive response above this should be to species-specific antigens, of organism common in the environment. On this basis, it is concluded that in Urumbrai, Puttur, and Hambanthota all the organisms that were used to prepare the skin test reagents were universally present. *M. vaccae*, *M. scrofulaceum* and MTB can be considered to be absent or only infrequently met in Darmakabeer. The high positivity to burulin needs further investigation. Studies on favourable ecological conditions for the growth of *M.ulcerans* suggests flooded poorly drained soils and the bog vegetation of some tropical and sub tropical areas as possible sources of infection (Kazda, 1983). Endemic areas for *M.ulcerans* infection are noted for their high temperatures and humidity, a type of climate, which is found in both Jaffna and Hambanthota. Tall spiny grasses like *Echinocola pyramidalis* have been suggested as the mode of introduction of the organism into the skin in Uganda, where buruli infection is endemic (Lema and Stanford, 1984). The study of vegetation ecology of the areas investigated would, at least to a certain extent, explain the observed high positivity for burulin.

In Hambanthota, BCG enhanced positivity only a little, whereas in Jaffna skin test responses were greatly enhanced by BCG vaccination. This increased response in Jaffna could be simply due to cross reactivity to shared antigens. The cross

reactivity could have been ruled out if the *M.ulcerans* is absent in this environment as it was thought. The degree of correlation between sensitisation to tuberculin and other skin test reagents prepared from environmental mycobacteria amongst the unvaccinated population can also reflect the level of cross reactivity between the antigens. However, this approach could not be utilised in this study, as the number of unvaccinated subjects was very low.

Compared to Jaffna, there were a high number of category 2 (anergy) reactors amongst vaccinated children in Hambanthota, 20% versus 49% respectively. There are several explanations for the lack of skin test response of category 2 individuals. In children, naivety, i.e. not yet having developed a response to mycobacteria, is the explanation in many cases. This explanation is not applicable to the present study population; as such a large proportion of the category 2 individuals had been vaccinated.

Non-responsiveness may also occur through recognition of suppresser determinants in the new tuberculins. Nye and colleagues demonstrated the presence of suppressive factors amongst species-specific antigens (Nye, *et al.*, 1983). They showed that these substances work in such a way that they not only suppress the responses to other co-injected mycobacterial antigens but also block responses to these other antigens injected simultaneously, at a distant site. The level of threshold of recognition of such suppressers can vary in individuals. In the present study, it cannot be the explanation since this occurred only in Hambanthota.

The high proportion of lack of responsiveness therefore may be associated with the lack of the HLA-DR3 (van Eden, *et al.*, 1983), gene, which encodes MHC class II. This plays an important role in presenting antigens to T-cells and initiating the subsequent delayed hypersensitivity reaction. This raises the question whether there is any association between the absence of HLA-DR3 and the ethnicity, because the study population in Jaffna were Tamils and in Hambanthota were Singhalese and Muslims. However, no significant difference was found either in percentage of reactors or reaction size of Singhalese and Muslims in the Hambanthota district. This suggests the lack of responsiveness

was not linked to genetic variations that occur between Singhalese and Muslims. Conclusions could not be drawn regarding the association of HLA-DR3 with Tamils and Singhalese as these two populations were from different geographical locations.

Reaction to the tuberculin skin test in an unvaccinated population in a given area is a good measure to determine the risk of infection in that location. The results for tuberculin in unvaccinated children (Fig 3.3) show contact with MTB was very low in Hambanthota compared to Jaffna. The further evidence for this is, that tuberculin conversion even amongst vaccinated children in association with increasing age was not remarkable in Hambanthota compared to Jaffna, per cent increase in association with increasing age is 16% in Hambanthota and 35% in Jaffna (Table 3.6). It is not clear whether the reduction in the tuberculin conversion rate in Hambanthota is due to the low prevalence of MTB in this area or to genetic resistance to tuberculosis. Inhabitants of Jaffna are Tamils, and in Hambanthota are Singhalese and Muslims, descendants of Arab traders. Several lines of evidence point to host genes controlling resistance or susceptibility to TB (Davies and Grange, 2001). A single dominant autosomal gene *bcg* has been identified in mice that controls resistance to TB (Gros, *et al.*, 1981). This gene has been found to code for a membrane transport protein, which is a homologue to an eukaryotic transporter. This finding may be related to the importance of nitric oxide in macrophages, essential for killing of intracellular pathogens. A human homologue of this gene has now been identified (Bellamy, 2000). This may be one of the reasons for the resistant nature found in Singhalese and Muslims. On the other hand, it cannot be concluded that the susceptible nature of the Jaffna children is due to the absence of *bcg* gene in them, because the susceptibility observed may be due to prevalence of different environmental mycobacteria, habits and nutritional intake in that area.

The resistant nature of the Hambanthota inhabitants may be linked to the daily diet. The main source of income in Hambanthota is fishing, whereas it is farming for inhabitants of Urumbrai and Puttur. Therefore, consumption of fish is high in Hambanthota compared to Jaffna, where meals are mainly made with vegetables. Epidemiological evidence suggests a link between vitamin D

deficiency and susceptibility to tuberculosis (Davies, 1985, Rook, 1988). Vitamin D is obtained from food sources and is manufactured in the skin through the action of sunlight. The main dietary sources of vitamin D are fish and fish liver oil. Therefore, consumption of fish in the daily diet of Hambanthota inhabitants should have rendered these children more resistant to tuberculosis infection. The active metabolite of vitamin D, 1,25-dihydroxyvitamin D3 (1,25-dihydroxycholecalciferol), activates monocytes, stimulates cell-mediated immunity, and enhances the ability of human monocytes to resist the intracellular growth of MTB (Bellamy, 2000).

When the overall reaction size to tuberculin was compared in vaccinated and unvaccinated children no significant difference was found. One of the previously published works also showed that naturally occurring tuberculin positivity was associated with responses larger in size than those following BCG (Aronson and Aronson, 1953).

The tuberculin skin test also allows the identification of latently infected people. The choice of cut-off reading on which to base the recommendation for treatment of latent infection has to be determined according to the local epidemiological situation of TB infection. The American Thoracic Society has recently provided new guidelines for targeted tuberculin testing and treatment (2000). According to these recommendations, which are based on the specificity and the sensitivity of the PPD, a reaction of 5 mm or more is considered positive for latent infection for those who are at high risk of developing active TB infection such as HIV-infected individuals. People with a reaction size of 15 mm or more are considered latently infected if they are at locations where the risk of TB infection is low. Sri Lanka can be grouped under the low risk category as the incidence of both pulmonary and extra-pulmonary TB infection has remained at about 50 per 100, 000 population for the past several years (Uragoda, 1996). This is similar to the TB incidence in Singapore (Chee, *et al.*, 2001). A recent study done on Singaporean school children recommended treatment for latent tuberculosis infection in those eliciting a reaction size of 10 mm or more and vaccinated at birth. However, a larger reaction size of 16 mm or more was recommended for latent treatment in those who have received a second BCG vaccination at an older age (Chee, *et al.*,

2001). Based on these recommended cut-off values (10 mm for those vaccinated at birth), it is concluded that about 40.5% of children in Jaffna are latently infected whereas only 7.3% are infected in Hambanthota and Darmakabeer Mahavidhiyalaya respectively.

The effect of BCG on responsiveness to species other than MTB is variable at both geographical locations. The reactivity to new tuberculins amongst 6-10 year olds has not been affected by the BCG vaccination. In addition, a low frequency of positivity to tuberculin was observed amongst unvaccinated 6-10 year olds. This suggests that the age of seven, which is the school entry age in Sri Lanka, would be a suitable time for BCG vaccination.

Re-vaccination of children in Hambanthota resulted in a significant increase in positivity to tuberculin, and to all the other skin test reagents except scrofulin (Fig 3.10). The increase in reactivity to tuberculin after the second vaccination was much more than that was found due to the passage of time alone in children vaccinated at birth. A similar observation was made in Kuwaithy children who received their first vaccine just before starting school (Shaaban, *et al.*, 1990). In a large proportion of children, the reaction sizes were larger after revaccination than after a single vaccination (Fig 3.8). However, the average mean induration of vaccinated and re-vaccinated positive reactors to tuberculin were  $7.67 \pm 3.8$ , and  $8.7 \pm 3.1$  respectively and there was no significant difference between these values. Very large average mean induration reaction sizes have been reported in children with two BCG scars (Ildirim, *et al.*, 1995). This is a disadvantage with revaccination, as large tuberculin reaction may cause confusion and undervalue the diagnostic value of tuberculin skin test. However, in the present study population, the average mean induration size of positive reactors for tuberculin was only 8.7 mm 1-2 years after the second vaccination. It should be noted that this re-vaccinated population is from Hambanthota, where reactivity to skin test reagents was very low even in the BCG vaccinated children.

As with a single BCG vaccination, an increase in reactivity was expected after the second vaccination in association with increasing age. However, neither the

percentage of positivity nor the reaction size increased for tuberculin skin test due to the passage of time after the second vaccination.

In conclusion, BCG can be considered effective in Sri Lanka, as it has increased species-specific skin test positivity among vaccinated school children. Geographical variations and dietary habits significantly influenced the reaction to tuberculin and new tuberculins. It is certain from the present study that ethnic differences, at least between Singhalese and Muslims, did not significantly affect the responsiveness to tuberculin and the new tuberculins. Furthermore, even though waning of response was observed in the reactivity at age of five in children vaccinated at birth in Sri Lanka (Karalliedde, *et al.*, 1987), according to the present study the recognition of antigens picked up by the age of eleven. A second vaccination did increase the recognition of antigens but did not increase it further over time. However, the increase in tuberculin responsiveness and enhanced recognition of environmental mycobacterial species indirectly support the view that a second vaccination improves the protective immunity.

## **Chapter 4**

### **4. Evaluation of immunoglobulin (Ig) response to a panel of mycobacterial antigens in healthy, tubercular and latently infected people**

#### **4.1 Evaluation of diagnostic value of measuring IgG antibody using ELISA to the 16kDa recombinant antigen of MTB in the institutionalised elderly persons**

##### **4.1.1 Introduction**

The geriatric population among all ethnic groups and both genders represent the largest reservoir of TB infection, particularly in developed nations (Davies, 1997, Davies, 1999). They are at greater risk for developing active disease because of age-associated decline in immune response to infectious agents. Ageing of the immune system is a complex process involving both humoral and cellular-mediated immunity. Along with declining immunity, underlying acute or chronic diseases, malnutrition, and the morphological changes in various organs make the elderly especially vulnerable to infection. The subtle clinical manifestation of TB disease in the elderly often makes diagnosis difficult. Chest X-ray changes may also mislead the clinicians, as disease is frequently present in the mid or lower zones (Patel, *et al.*, 1993). Unfortunately, this treatable infection is often recognized only at autopsy.

Compared to community-dwelling elderly, the institutionalised elderly are at greater risk for reactivation of latent TB as well as for the acquisition of new TB infection. The TB case rate is two to three times higher among the elderly in nursing homes than in comparable persons living at home. Stead and colleagues found a 5% rate of tuberculin conversion in an institutionalised elderly population who were living with a known recent infectious case. They also noted a 3.5% rate of tuberculin conversion for each year among elderly in a nursing home where no recognised recent case was found (Stead, *et al.*, 1985). It is likely that the stress of moving to an institution is responsible for the immune changes leading to reactivation and to susceptibility.

The diagnosis of latent tuberculosis infection still largely depends upon the tuberculin skin test. The significance of the tuberculin skin reaction in the elderly remains rather uncertain. Some have contended that most elderly persons will have positive tests because of earlier infection, whereas others have argued that most will have negative tests because reactivity to tuberculin decreases with age (Dorken, *et al.*, 1987). Nevertheless, a study conducted on a very large elderly population has demonstrated a high incidence of TB among untreated reactors to tuberculin. While 3% of untreated reactors developed TB, only 0.02% of non-reactors developed TB (Stead and To, 1987), suggesting the clinical importance of the skin test in the diagnosis of LTBI.

Despite its extensive use for nearly a century, the cross-reactive nature of the PPD undervalues the diagnostic value of the tuberculin skin test. Therefore, in recent years, the immunological response to species-specific antigens has been extensively investigated in order to develop a serodiagnosis method with increased specificity and sensitivity for latent tuberculosis infection and for active disease.

The alpha-crystallin protein of the MTB complex is one of those species-specific antigens, known mainly for its expression under hypoxic conditions and during late log phase *in vitro* (Yuan, *et al.*, 1996). This antigen contains B-cell epitopes (Verbon, *et al.*, 1992). The immunodominance of this antigen has been demonstrated in both human and murine investigations (Jackett, *et al.*, 1988, Friscia, *et al.*, 1995) (Wilkinson, *et al.*, 1998) (Imaz, 2001). Selectively raised antibody positivity for the 16kDa antigen has been described in self-healed tuberculosis (Bothamley, *et al.*, 1988), in tuberculosis case contacts (Jackett, *et al.*, 1988, Imaz, 2001), after heavy occupational exposure (Bothamley, *et al.*, 1992a), and in the cerebrospinal fluid of patients with tuberculous meningitis (Chandramuki, *et al.*, 1989). All these data suggest a selective sensitisation against this antigen in the early stage of infection. Interestingly, increasing antibody titre was found for this antigen following chemotherapy, but low levels have been associated with particularly severe disease and poor prognosis (Bothamley, *et al.*, 1992a). This suggests *in vivo* hypoxic shiftdown of tubercle bacilli to the non-replicating state after chemotherapy as demonstrated in the *in*

*vitro* bacteriological model (Wayne and Sohaskey, 2001) and persistence of bacilli.

*In vitro* studies have described up-regulation of 16kDa in MTB that shift towards a non-replicating state under hypoxic conditions (Wayne and Hayes, 1996, Yuan, *et al.*, 1996). According to the widely accepted immunopathogenesis of TB, MTB adapt to the hypoxic condition *in vivo*, thereby surviving there for many years without causing disease. As reactivation of tuberculosis is the main source of TB in the elderly, it is postulated that latent infection should be highly prevalent among them.

Therefore, this study has attempted to investigate differences between elderly and healthy individuals in terms of prevalence of anti-16kDa antibody levels and any relationship to delayed type hypersensitivity (DTH).

The most widely evaluated species-specific antigen in serum immunoassay in active infection is 38kDa, also known as antigen 5. Independent groups have consistently demonstrated a sensitivity of 70% or more with a specificity of 95% for this antigen when testing sera from proven tuberculosis patients and controls (Bothamley, *et al.*, 1998). This suggests sensitisation to this antigen is associated with active disease. Therefore, the ratio of serum level antibody titres of 16kDa: 38kDa would be expected to be higher in latently infected people than in active TB cases.

In this study, indirect enzyme-linked Immunosorbent assay (ELISA) was employed to measure the antibody titre to recombinant 16kDa and 38kDa antigens of MTB, with the objective of testing the above-mentioned hypothesis. Sera were obtained from healthy people from TB high and low endemic countries; from TB patients before starting the treatment; and from institutionalised elderly persons from Spain.

In addition to the 16kDa and 38kDa antigens, antibody responses to two other heat shock proteins (HSPs), 65kDa and 70kDa were also studied. This was to ascertain the reactivity due to the recognition of shared antigens. Evaluation of

correlations between the antibody titre against various antigens in people from different locations should rule out possible cross reactivity between those antigens. Members of the HSP70 and HSP60 families, (70kDa and 65kDa respectively) play an important role when a cell is heat-shocked, in preventing disassembly of oligomeric complexes and the unfolding of polypeptides (Kaufmann, 1990). HSPs are major antigens of many pathogens. High homology exists between the 65kDa antigen of MTB and humans. Therefore, the detection of antibodies against this antigen cannot be taken as an indication of infection with MTB.

The major objectives of the study in this chapter were:

To evaluate the diagnostic value of the IgG response to the 16kDa antigen as an immunoassay for identification of latently infected people with tuberculosis;

To study the relationship between the delayed type hypersensitivity response to the skin test and the immune response to 16kDa and 38kDa antigen in elderly persons; and

To evaluate the proportion of antibody titre to the 16kDa and 38kDa antigens in TB patients and latently infected people.

#### **4.1.2 Materials and methods**

The indirect ELISA technique was employed to detect the immunoglobulin G titre in sera obtained from healthy people, TB patients, and elderly persons. Experimental details are described in chapter 2.

##### **4.1.2.1 Antigens**

Recombinant MTB antigens, 16 kDa and 38kDa, were obtained in powder form from Lionex Diagnostic and Therapeutics, Germany and lyophilised in PBS prepared with non-pyogenic water. Antigen 65kDa of *M.bovis* BCG and antigen 70kDa of MTB were provided by Prof. John Stanford.

#### **4.1.2.2 Subjects**

- 1) Thirty five elderly people were recruited into this study from the geriatric unit at Sanatorium San Francisco de Borja in Fontilles, Spain. They had been in the sanatorium for more than one year. All were tuberculin skin tested with new tuberculin, equivalent to 2 tuberculin units per 0.1 ml. The skin test was administered by the Mantoux technique into the skin of the volar surface of the forearm. Reactions were read at 72 hours; diameters of the induration along the longitudinal and cross axis were measured and the average of this measurement was used in the study. The skin testing was done by Dr. P. Torres, who is well experienced in this technique. The participants age ranged from 60 to 93 years.
- 2) Eighteen serum samples were collected from HIV-negative patients with either smear or culture positive active pulmonary TB, before starting therapy. The ethnic origins of these TB patients differed but they all resided in the UK.
- 3) Sera from 36 healthy Spanish people, and 36 Londoners were used as negative controls for sera from geriatric persons, and UK TB patients, respectively. Also, sera from 41 Sri Lankans, children/young adults and 22 sera from Mexicans (obtained from blood bank) were tested for antibody titre against each of the antigens evaluated in this study.

#### **4.1.3 Statistics**

Correlations were analysed by linear regression, and Pearson's non-parametric independent variable correlation and mean values were compared using the Mann-Whitney test.

#### **4.1.4 Results**

The average age of the elderly persons included in this study was  $77.2 \pm 8.4$  years. The BCG vaccination and the past history of tuberculosis infection in this group is not known. They had not been tested for tuberculin positivity since they were admitted into the Sanatorium. Among the total tested, 37% were negative for the tuberculin skin test; small reactions were relatively infrequent: 20% of the

group showed a reaction size of 1 to 9 mm. A reaction size greater than or equal to 10 mm was elicited in 43%.

IgG titres in sera from healthy people, TB patients, and elderly people against each of the four antigens tested are shown in Figure 4.1. Sera from healthy Spanish people were included as controls for the Spanish elderly group and sera from Londoners were used as controls for UK TB patients. A significantly greater IgG response to all four antigens was observed in elderly people compared to healthy Spanish subjects. However, the antibody titres in the elderly group against the 16kDa and 38kDa antigens were as high as in TB patients. Nevertheless, antibody titres against the 65kDa and 70kDa antigens were significantly higher in the elderly group compared to TB patients. A significant increase in the IgG titre was found only to the 38kDa antigen in TB patients compared to healthy controls. There was no significant difference in the IgG levels to any of the four antigens among healthy people from different countries.

ELISA measurements of serum antibody against 16kDa and 38kDa antigens in TB patients, elderly people, and appropriate healthy controls are shown in Figure 4.2. Antibodies against the 16kDa antigen were detected in 43% and 16%, of geriatric persons and TB patients respectively. The 38kDa antigen was recognised by sera from 29% of geriatric subjects and 28% of TB patients.

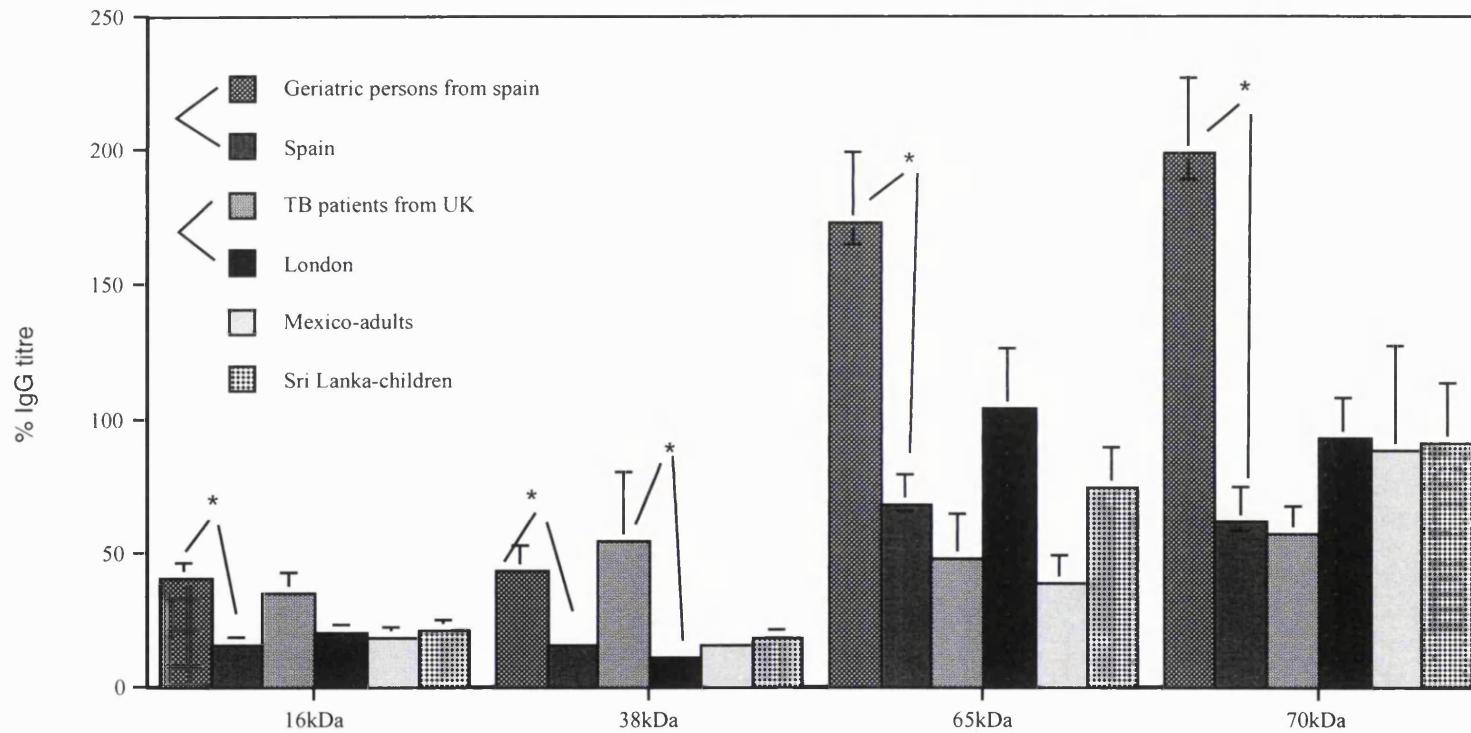
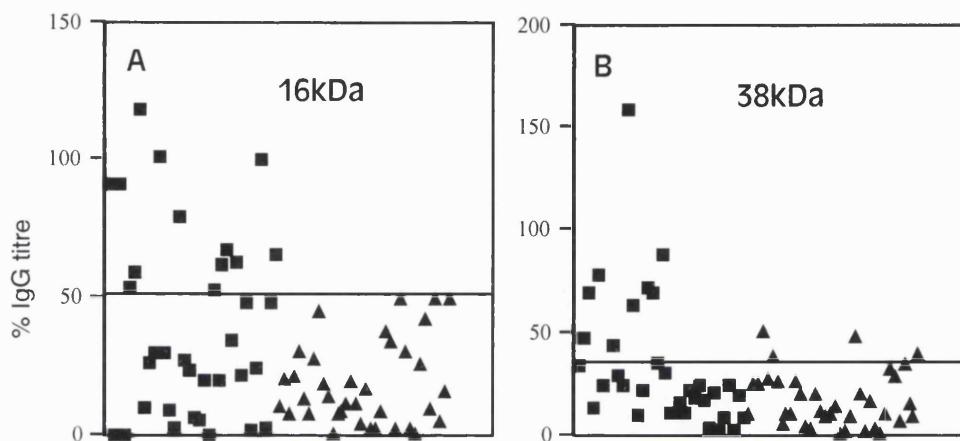
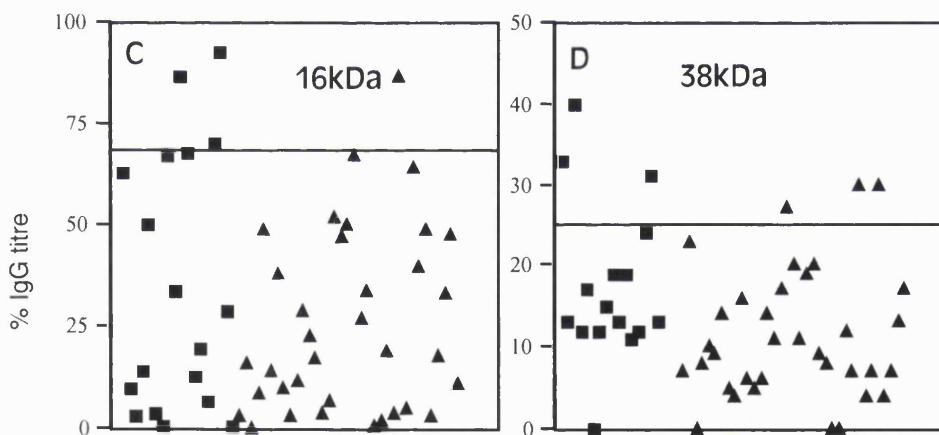


Figure 4.1: Mean values ( $\pm$ standard error mean) of anti-16, 38, 65, and 70kDa IgG levels in individuals according to the source country, geriatric persons, and TB patients are shown. Sera from healthy people from Spain and London were used as controls for geriatric persons from Spain and TB patients from UK, respectively. The IgG levels for different antigens should not be directly compared as different dilutions of the sera were used with different antigens

\* $<0.05$ ; Significantly different



IgG seroreactivity to 16kDa antigen (A) and 38kDa antigen (B) in Spanish elderly persons (squares) and healthy controls from Spain (triangles). Cutoff points (mean absorbance plus two standard deviations of healthy controls) of seropositivity was 51 for 16kDa and 43 for 38kDa.



IgG seroreactivity to 16kDa antigen (C) and 38kDa antigen (D) in TB patients from UK (squares) and healthy controls from London (triangles). Cut off points (mean absorbance plus two standard deviations of healthy controls) of seropositivity was 71 for 16kDa and 26 for 38kDa. (Seroreactivity of two TB patients to 38kDa antigen has not been shown as they were very high.)

**Figure 4.2: IgG antibody titre to 16kDa and 38kDa antigens**

Among the geriatric population, no linear correlation was found between the reaction size to tuberculin and levels of either anti-16kDa or anti-38kDa antibodies.

No consistent correlation pattern was observed between antibody titres to different antigens amongst healthy people from each of the different countries (Table 4.1). Significant correlations were found between antibody titres to each of four antigens in Mexicans, Spaniards, and Londoners. In contrast, amongst the Sri Lankan sera from children tested, no positive correlation was found between the antibody titres to any of the four antigens evaluated in this study. A negative correlation was found between anti-38 kDa and 65kDa antibody titres. Apart from the correlation between anti-16kDa and 65kDa antibodies, no correlation was observed between the other antibodies of TB patients. Among the elderly group, significant correlations were found between anti-16kDa/anti-38kDa, anti-16kDa/65kDa and anti-65kDa/70kDa antibodies.

	Geriatrics' sera	TB patients' sera	Spanish sera	Sri Lankans' sera	Mexicans' sera	Londoners' sera
Anti-16/38kDa	$r = 0.51$ $P < 0.0001$	NS	$r = 0.34$ $P < 0.035$	NS	$r = 0.53$ $P < 0.012$	$r = 0.37$ $P < 0.031$
Anti-16/65kDa	$r = 0.38$ $P < 0.023$	$r = 0.69$ $P < 0.003$	NS	NS	$r = 0.51$ $P < 0.017$	$r = 0.38$ $P < 0.03$
Anti- 16/70kDa	NS	NS	NS	NS	NS	NS
Anti-38/65kDa	NS	NS	$r = 0.49$ $P < 0.002$	$r = -0.44$ $P < 0.046$	$r = 0.65$ $P < 0.001$	$r = 0.46$ $P < 0.005$
Anti- 38/70kDa	NS	NS	$r = 0.57$ $P < 0.0001$	NS	$r = 0.60$ $P < 0.03$	NS
Anti-65/70kDa	$r = 0.64$ $P < 0.0001$	NS	$r = 0.74$ $P < 0.0001$	NS	$r = 0.80$ $P < 0.0001$	$r = 0.48$ $P < 0.004$

**Table 4.1: Correlation between levels of antibodies against all four antigens in healthy people, TB patients and geriatrics**

NS Not significant ( $p > 0.05$ )

#### 4.1.5 Discussion

The manifestation of disease with *M. tuberculosis* in a latently infected person depends upon any imbalance that occurs between the bacillus and the host defence mechanisms. The chance that the geriatric population in the present study is latently infected is high, as they have lived through times when TB was highly prevalent in their communities. Profound and complex changes in the immune response occur during the aging process. The most dramatic immunological effect of aging is on cell-mediated immunity, which plays an important role in tackling intracellular pathogens, and there are several pieces of evidence, which suggest that TB amongst the elderly persons in a low prevalence community is mainly due to reactivation (Rajagopalan and Yoshikawa, 2000b).

The two most prominent features of immunosenescence are altered T cell phenotype and reduced T cell response. One of the most consistent changes

noted in the T cell population of the elderly is the increase in memory T cells with a concomitant decrease in the proportion of naive T cells (Chandra, 1990). This is because of the age-associated decline in the thymus function, which plays an essential role in T cell maturation. Amongst the geriatric population in this study, 37% were negative for the tuberculin skin test. This may be caused by anergy to tuberculin, which has been shown to increase with age (Dorken, *et al.*, 1987) or to delay in recalling the memory T-cells (Stead and To, 1987). Orme has studied the immune responsiveness in old mice and concluded that the increased susceptibility to tuberculosis of old mice is due to a limited ability by CD4 cells to accumulate at the sites of the bacterial implantation (Orme, 1987). Other studies have also shown a reduced expression of homing molecules such as L-selectin and CD11a on CD4 cells, which are important in allowing lymphocytes to cross inflamed endothelial surfaces (Marlin and Springer, 1987). Therefore, the inability to undergo trans-endothelial migration and accumulation at the site of inoculation of skin test reagent may explain the anergy observed in the elderly. Castle and colleagues studied peripheral blood mononuclear cells (PBMC) of the frail elderly in comparison with PMBC's from young control subjects, and reported an enhanced type 2 immune response and impaired type 1 response in the elderly group (Castle, *et al.*, 1997). Th1 cells are required for the DTH response, as the inflammation is mediated by IFNy. Therefore, another possible cause of anergy in the elderly may be the lack of Th1 type response. Various studies have shown that re-testing of those negative for the tuberculin skin test with the same amount as the first dose can recall the memory T cells and lead to a reaction to the test (Stead, *et al.*, 1985, Stead and To, 1987).

A skin reaction of 10 mm or more in the tuberculin skin test is highly suggestive of current or previous infection. If this is indeed the case, 40% of the geriatric population of this study are latently infected. None of these subjects showed any symptoms, of tuberculosis. Nevertheless, 14% of them elicited a reaction size of 15 mm or more, suggesting recent tuberculin conversion, as reactions due to old infections are generally smaller (Stead, 1991). However, a recent study done on the relationship between size of tuberculin reaction and active disease, and indicators of high risk for active disease in the future, showed that above the

threshold of 5 mm reaction size to 5-TU of PPD, the size of tuberculin skin test reactions had no meaning (Al Zahrani, *et al.*, 2000).

Grange and colleagues studied IgG, IgA and IgM responses to mycobacterial antigens in TB patients using ELISA and found that among the three immunoglobulins, IgG was the most discriminative (Grange, 1980). Therefore, the IgG response to the MTB-specific antigens 16kDa and 38kDa in geriatrics was investigated with a view to develop a serodiagnostic method for latent tuberculosis infection. As some variation (5-10%) was observed between each plate even when prepared on the same day, four standard control sera were included in each plate and all the optical densities were standardised according to the control (see chapter 2). Further reliability was obtained by not using the outer wells on the plates.

Antibody titres to 16kDa and 38kDa antigens were higher in geriatrics than in healthy controls ( $p= 0.01$  for 16kDa and  $p=0.004$  for 38kDa) (fig 4.1). Selectively raised antibody levels to the 16kDa antigen have been described previously in people likely to harbour tubercle bacilli without clinical symptoms (Bothamley, *et al.*, 1988, Bothamley, *et al.*, 1992a). All these data agree with the hypothesis that the sero-reactivity to the 16kDa antigen can be a marker for latent tuberculosis infection. However, the raised sero-reactivity to the 38kDa antigen in geriatrics is intriguing, as a raised antibody titre to this antigen has previously been reported in many studies of active pulmonary patients (Bothamley, *et al.*, 1998). Therefore, an inverse relationship was expected between anti-16kDa and 38kDa antibody levels in geriatric subjects and TB patients. However, this study has demonstrated a positive correlation between the antibody titres to these antigens in geriatric subjects (Table 4.1), indicating the elicitation of a parallel humoral response to these antigens. It is not clear, however, whether the sero-reactivity to these antigens was due to specific B cell expansion, i.e. an oligoclonal response.

While the titre of antibodies to foreign antigens declines with age, the prevalence of autoantibodies increases in the elderly (Haeney, 1994). This supports the observation of high titres of IgG for the 65kDa and 70kDa antigens found in the elderly population of this study compared to healthy controls and TB patients (Fig

4.2). In addition, a linear correlation was found in between the anti-65kDa and 70kDa antibodies. Increased antibody levels to heat shock proteins (HSP) have been observed in association with various autoimmune diseases, which are usually common in the elderly: these include antibodies to 65kDa in rheumatoid arthritis, and to 70kDa in lupus erythematosus (Bahr, *et al.*, 1988) (Tsoulfa, *et al.*, 1989). Furthermore, HSPs are major antigens of many pathogens, including helminths, protozoa, and bacteria (Young, *et al.*, 1987). Life long exposure to various antigens and previous subclinical infection with a variety of microorganisms could also have induced a certain level of humoral response to these antigens in the elderly.

Sensitivities of 38% and 31% were obtained by ELISA in TB patients for 16kDa and 38kDa antigens, respectively. Well-controlled trials have shown that the 38kDa antigen is the best reagent for serodiagnosis of active tuberculosis. The low sensitivity observed in the present study may be due to the small number of samples used. Sera from 18 active TB cases, either culture- or smear- positive were tested. Two of them were found to have very high anti-38kDa antibodies and therefore excluded from the statistical analysis, as outliers can affect the final result. This suggests heterogeneity of antigen recognition among TB patients. Multiple factors can cause the heterogeneity of antigen recognition: 1) Immunogenetic background of the infected host; for humans, an association between antibody titres against particular MTB specific epitopes and certain HLA (Human Leukocyte Antigen) alleles has been described (Bothamley, *et al.*, 1989). The TB patients in this study were of different ethnic origin. 2) The second factor in heterogenous antigen recognition may be the production of different mycobacterial antigens at different stages of tuberculosis. For example, a high antibody titre to the 38kDa antigen was found after commencing treatment (Bothamley, *et al.*, 1992b). Sera from TB patients investigated in the present study were obtained before the commencement of treatment.

Evaluation of the antibody response to each of the four antigens in sera obtained from various geographical locations has shown no significant difference, suggesting individuals were not specifically sensitised to any of the antigens tested.

This is the first study of the correlation between the antibody response to various antigens in healthy subjects from different geographical locations. The correlation pattern between antibody responses varied according to geographic areas, indicating the absence of cross-reactivity between antigens. Within the healthy control sera, except for the sera from Sri Lanka, a significant correlation was found between the antibody responses to various antigens (Table 4.1). The sera from Sri Lanka were obtained from children. These findings suggest that any significant correlation between antibody responses is due to parallel humoral responses, which may occur because of long-term exposure to various antigens in the past.

Compared to sera from healthy controls, the frequency of correlation between antibody responses to different antigens was low in TB patients and geriatrics, suggesting elicitation of a specific humoral response to specific antigens rather than a non-specific parallel humoral response as in healthy controls.

Interestingly, a positive correlation was found between anti-16kDa and 65kDa antibodies in both geriatrics and TB patients. Immunoglobulins may be initially produced to neutralise mycobacterial HSPs secreted by multiplying cells. As the HSPs of mycobacterial origin display epitopes resembling the host counterparts, they could induce an immune response which breaks tolerance to the host's own molecules (Munk, *et al.*, 1989). Therefore, immunoglobulins initially produced against mycobacterial HSPs could cross-react with the host's own structures resulting in a pathogenic autoimmune response. The host's regulatory attempts to reduce such an autoimmune response could suppress the reactivity to microbial HSPs. This could result in persistence. Persisting MTB up-regulate 16kDa antigen expression, and subsequently sensitise the host against this antigen. The same phenomenon can be applied to geriatrics, as autoantibodies are frequently present in this population.

There was no relationship between either anti-16kDa antibody levels or 38kDa antibody levels and the diameter of the tuberculin response. Similar observations were made in a study done on health care workers who have had prolonged exposure to TB (Bothamley, *et al.*, 1992a). As discussed earlier, reactions to the

tuberculin skin test are controlled by multiple factors. Impairment in the activation and recruitment of cells into the inoculation site could have caused the anergy to tuberculin reaction. Therefore, in any event, it is impossible for the anti-16kDa and 38kDa antibody levels to positively correlate with the tuberculin skin test reaction.

In conclusion, even though the antibody response to the 16kDa antigen was raised in geriatrics compared to healthy subjects, the parallel humoral response to 16kDa and 38kDa antigens warrants further investigation. Evaluating the quality of the antibody response to these antigens may reveal whether there was a parallel sensitisation to 16kDa and 38kDa antigens in the elderly people.

## **4.2 Evaluation of oligoclonal IgG antibody responses using isoelectric focusing (IEF) to recombinant antigens of 16kDa and 38kDa antigens of MTB in tuberculous meningitis cases and in the institutionalised elderly**

### **4.2.1 Introduction**

Of all forms of tuberculosis, tuberculous meningitis (TBM) is the most devastating, especially in children. Delayed treatment of TBM is associated with a high mortality and serious nervous complications. Thus, early initiation of appropriate therapy is very important and for this a fast and specific test for diagnosis is essential.

The 'gold standard' for the diagnosis of any bacterial infection is isolation of causative organisms on culture. Nevertheless, culture of tubercle bacilli takes a long period of 6 to 8 weeks and one cannot wait for the culture report to start the treatment. Demonstration of tubercle bacilli in the cerebrospinal fluid (CSF) would also be an excellent way to confirm the tubercular aetiology, but unfortunately, the yield of tubercle bacilli on acid-fast staining is very low (Ahuja, *et al.*, 1994). Recent work using the polymerase chain reaction (PCR) and bacteriophages has been of value in the early diagnosis of TBM (Brisson-Noel, *et al.*, 1989, Wilson, *et al.*, 1993, Del Portillo, *et al.*, 1996, Wilson, *et al.*, 1997). However, the high costs and the necessary high level of technical expertise limit the application of these techniques in routine diagnosis. In addition, the requirement of a large volume of CSF limits its use.

Although cell-mediated immunity is of the utmost importance for the course and outcome of human tuberculosis infection, the humoral immune response also plays an important role (Glatman-Freedman and Casadevall, 1998). Previous clinical trials of MTB-specific antibody detection in CSF by ELISA demonstrated its clinical usefulness (Watt, *et al.*, 1988, Dole, *et al.*, 1989, Park, *et al.*, 1993, Srivastava, *et al.*, 1998). However, the passive transfer of antibody from serum to CSF limits the diagnostic use of CSF ELISA, especially in TB endemic areas where a large proportion of the population is serum positive for mycobacterial

antigens (Cho, *et al.*, 1995). Therefore, it is crucial to measure the antibody titre in the CSF as well as the matched serum sample to avoid false positives.

In addition, even though ELISA is a cheap, time-saving and clinically useful method for early diagnosis, unfortunately it gives minimal qualitative data in terms of type of clonal expansion. Among the antibodies produced against an antigen only a very small fraction of the total population is specific for any one antigen, representing the products of a limited number of clones of plasma cells, referred to as oligoclonal antibodies. The rest stimulates many B or T lymphocytes irrespective of antigen specificity, and are referred to as polyclonal antibodies (Abbas, *et al.*, 1997).

TBM is characterised by the intrathecal synthesis of oligoclonal IgG. An intensive humoral response within the central nervous system has been reported, using IEF, in association with intrathecal synthesis of oligoclonal immunoglobulins, (Kinnman, *et al.*, 1981).

Previous studies have used mainly crude extract of MTB as the antigen to detect the specific binding of oligoclonal antibodies in the CSF compared to serum (Kinnman, *et al.*, 1981, Sindic, *et al.*, 1990). In the present study, a panel of 10 mycobacterial antigens were used in a simple immunoblotting assay to explore the diagnostic value of these antigens in diagnosis of tuberculous meningitis. Antigens, to which an elevated antibody response was found in TBM patients, were then employed to study the intrathecal production of oligoclonal IgG, using the IEF technique.

The same technique used to study the intrathecal oligoclonal response in TBM patients was also employed to study the quality of the antibody response to 16kDa and 38kDa in the geriatric subjects studied in section 4.1.

#### 4.2.2 Materials and methods

##### **Subjects:**

Matched serum and CSF were obtained from the following subjects:

A 12-year-old girl with culture confirmed TBM, and three multiple sclerosis (MS) patients. Undiluted concentrated CSF and 1:400 diluted sera were used in the experiments.

CSF alone was obtained from the following individuals:

- 1) Twenty cases of TBM from Mexico with features characteristic of central nervous system infection (fever, and altered sensorium and convulsions, with or without headache, vomiting and neurological deficit) who had responded to antitubercular drugs.
- 2) Seven cases of culture-confirmed TBM cases from Britain.
- 3) A total of fifteen non-tuberculosis cases, seven samples from British patients and eight from Zambian patients.

Serum alone was obtained from the following:

- 1) Twenty seven institutionalised geriatrics

##### **Antigens:**

The following antigens were used in the screening of matched serum and CSF samples using a simple immunoblotting assay: 70, 38, 16,10kDa and MPT64 of MTB, 65kDa of *Mycobactrium bovis*; 65, 36, 28kDa of *Mycobacterium leprae*, and ultrasonicate of MTB. All other samples (CSF alone) were screened against only 16, 38, 70 of MTB, and 65kDa of *M. bovis*.

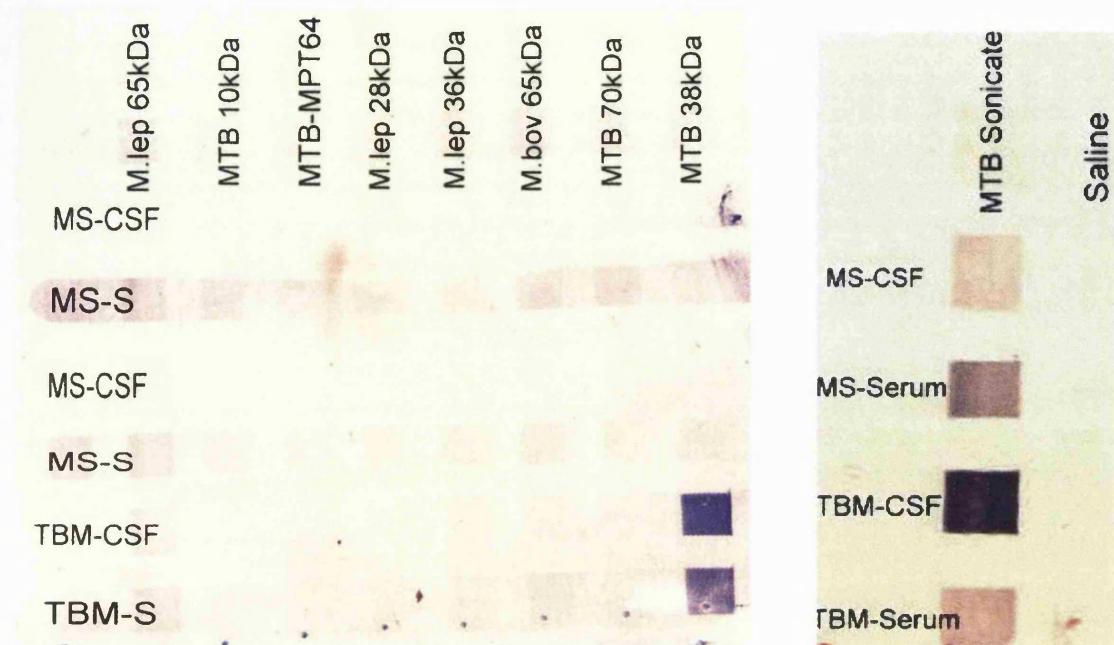
All the samples were focused by IEF and immunofixed to evaluate the total IgG pattern. Samples that reacted either with 16kDa or with 38kDa antigens were focused by IEF and immunofixed against both of the antigens.

### 4.2.3 Results

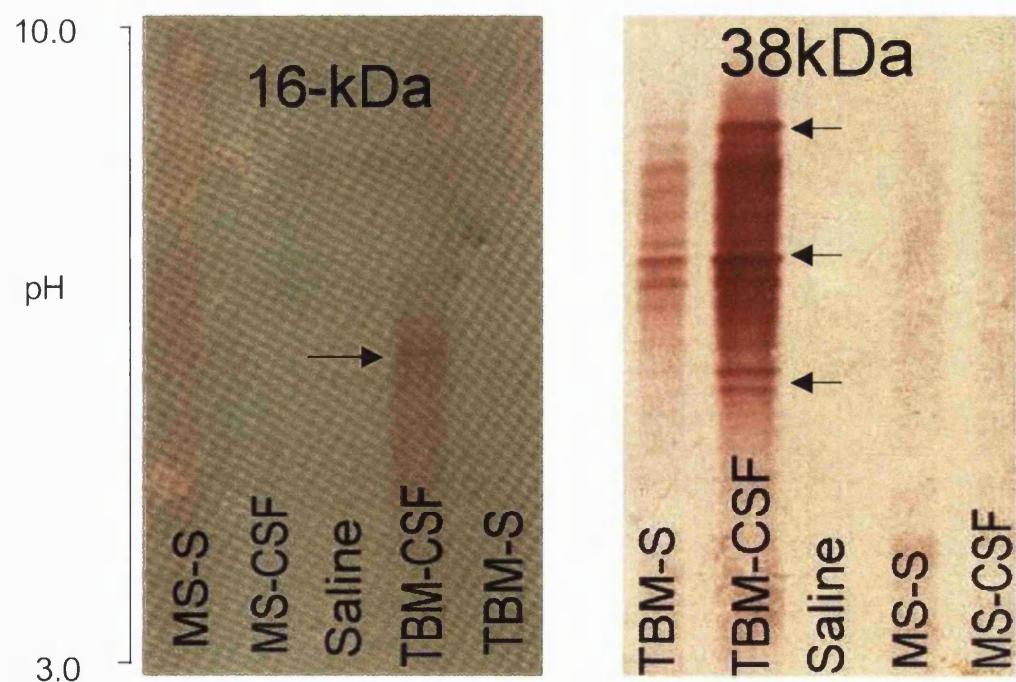
#### 4.2.3.1 Evaluation of IEF to measure oligoclonal antibody response in the CSF of TBM patients

Of the antigens screened against specimens from TBM and multiple sclerosis patients by immunoblotting, CSF and the matched serum of the TBM patient reacted very strongly only to 38kDa antigen (Figure 4.3) and very weakly to 16kDa antigen (data not shown). Even though an antibody response was found in CSF as well as in the matched serum of the TBM patient, the intensity of staining in the immunoblot was higher in the CSF compared to the serum, suggesting intrathecal synthesis of the antibody and passive transfer from CSF to serum. A non-specific reaction to various antigens was observed in the sera and CSF of multiple sclerosis patients. A strong antibody response to MTB ultrasonicate was found in both the TBM patient and in one of the MS patients.

CSF from the TBM patient revealed oligoclonal bands without any counterpart in serum against the 16kDa antigen (Figure 4.4). Both serum and CSF revealed oligoclonal bands against the 38kDa, however, the intensity was very high in the CSF, indicating local Immunoglobulin production against this antigen. None of the other CSF or serum samples revealed locally or systemically synthesised specific antibody to either of the antigens.



**Figure 4.3: Immunoblot of CSF and matched sera against a panel of antigens.** Representative picture of an immunoblot showing total antibody response to a panel of antigens in CSF and matched serum (S) of tuberculous meningitis (TBM) and multiple sclerosis (MS) patients.



**Figure 4.4: Pattern from IEF and subsequent immunofixation**  
Representative picture of pattern of IEF and subsequent immunofixation of 16 and 38kDa specific IgG of CSF and sera from the TBM and multiple sclerosis patients. Arrow heads denote oligoclonal IgG.

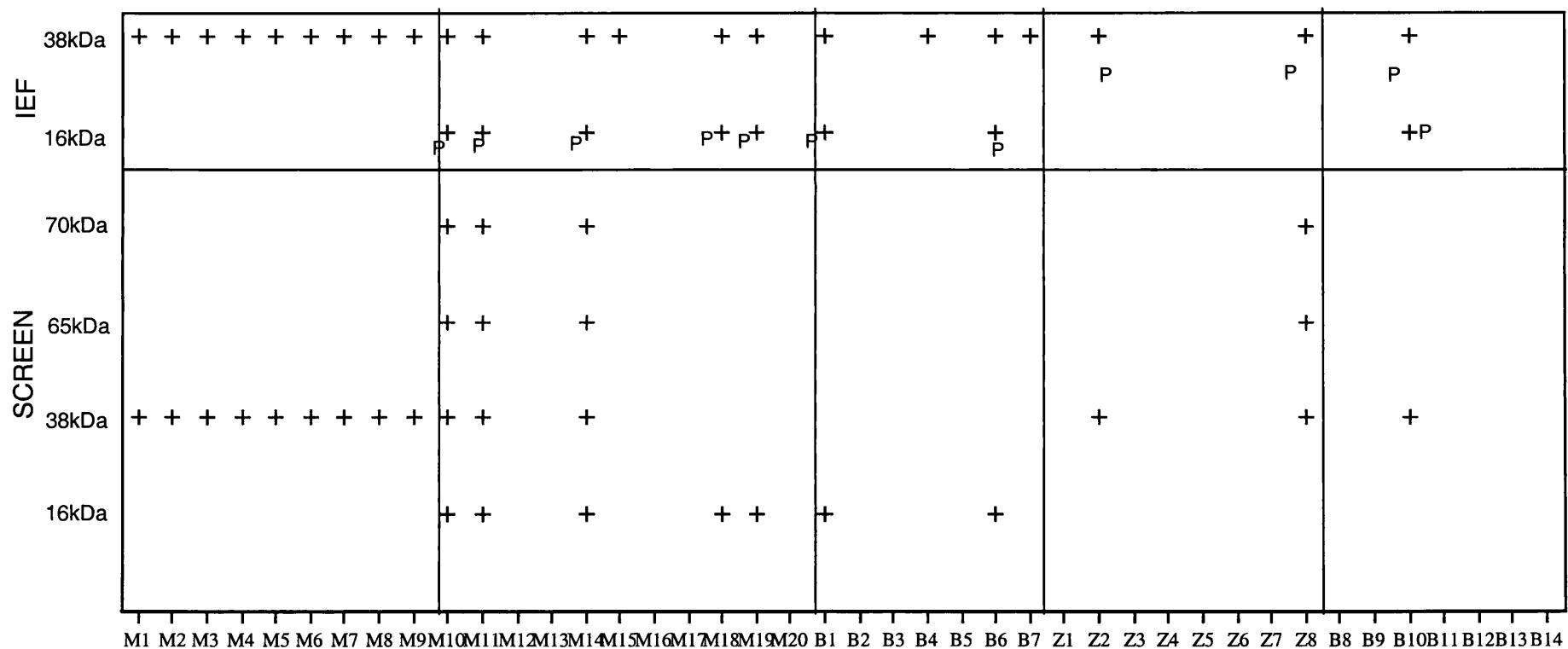


Figure 4.5: Overall result of immunoblotting screen and IEF of CSF from tuberculous meningitis patients and controls. +: Sera reacted to antigens. M1-M20: CSF from Mexican TBM patients; B1-B7: CSF from British TBM patients, Z1-Z8: CSF from non-TBM Zambian patients; B8-B14: CSF from non-TBM British patients. P: Shown only polyclonal response

**TBM**

Mexico	Screen	38kDa	12/20 (60%)
		16kDa	5/20 (25%)
	IEF	O	15/20(75%)
		38kDa	P
		O	
		16kDa	P 5/20 (25%)
	Screen	38kDa	0/7 (0%)
		16kDa	2/7 (29%)
UK	IEF	O	4/7 (57%)
		38kDa	P
		O	
		16kDa	P 2/7 (29%)

**Controls**

Zambia	Screen	38kDa	2/8 (25%)
		16kDa	0/8 (0%)
	IEF	O	
		38kDa	P 2/8 (25%)
		O	
		16kDa	P 0/8 (0%)
	Screen	38kDa	1/7 (14%)
		16kDa	0/7 (0%)
UK	IEF	O	
		38kDa	P 1/7 (14%)
		O	
		16kDa	P 1/7 (14%)

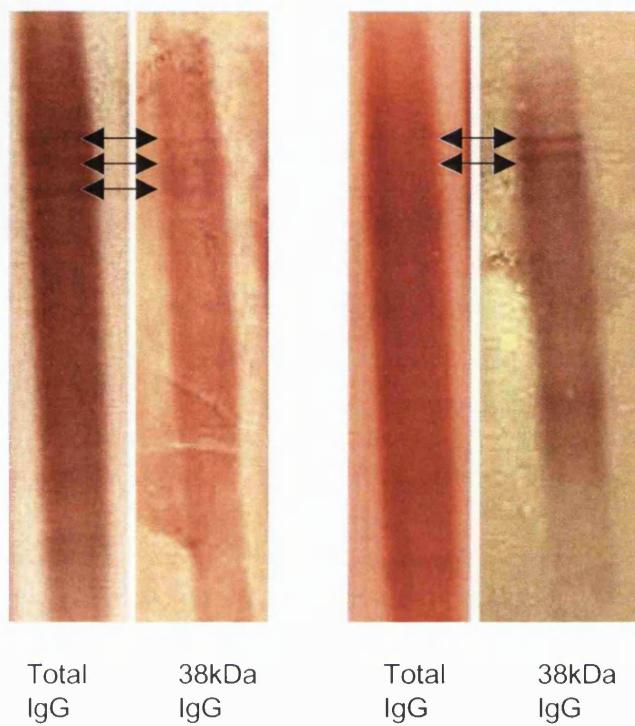
Table 4.2: Percentage of CSF samples reacted to 16 and 38kDa antigens.

O: Oligoclonal response; P: Polyclonal response

All the CSF samples without the matched sera were screened against 38, 16, and 70kDa antigens of MTB and 65kDa antigen of *M.bovis*. Three samples from Mexican patients (M10, M11 and M14) reacted to all the antigens used in the screening (Figure 4.5). Nine CSF reacted with 38kDa antigen and two (M18 and M19) to the 16kDa antigen (Figure 4.5). Two of the seven CSF samples from the British patients reacted with the 16kDa antigen and none reacted to the 38kDa antigen. Among the negative controls, two samples from the 8 Zambian patients, reacted to antigens tested in the screening. One of these reacted to all the antigens, except to the 16kDa antigen. A sample from non-tuberculosis British patient showed an antibody response against 38kDa.

The pooled data of the percentage of CSF samples reacting to 16 and 38kDa antigens are shown in table 4.2. Separation of the 27 CSF samples from TBM patients using IEF and subsequent immunofixation revealed oligoclonal bands in 19 of them to the 38kDa antigen. These included all of the 12 samples positive to this antigen in the screening procedure. Three samples from Mexican patients and four from British patients that lacked reactivity against the 38kDa antigen in screening procedure revealed an oligoclonal response to this antigen. Seven CSF samples from TBM patients that reacted to the 16kDa antigen in the screening procedure revealed only a polyclonal response against this antigen. CSF samples from the non-tuberculosis patients that reacted to the 38kDa antigen in the screening procedure revealed only a polyclonal response using IEF

When the total IgG patterns were compared with those of the anti-38kDa antibody IEF pattern (Figure 4.6), in eleven of the CSF from TBM patients, identical bands for pronounced bands in the total IgG were found in the immunoblot of 38kDa antigen.



**Figure 4.6: Representative picture of pattern of IEF and subsequent immunofixation of total IgG and 38kDa specific IgG.**

The arrowheads indicate identical location of oligoclonal bands of anti-38kDa antibodies in the total IgG IEF pattern

#### **4.2.3.2 Evaluation of IEF to detect oligoclonal antibody response in the sera of geriatrics**

Sera from 27 geriatric subjects showing seroreactivity to the 16kDa and 38kDa antigens, respectively, by ELISA were analysed by IEF and by subsequent immunofixation, in order to study the prevalence of oligoclonal antibody responses. It should be noted here that a positive correlation was observed between the antibody titres for 16 and 38kDa antigens in these geriatric subjects (Table 4.1). As the colorimetric method was not efficient enough to detect the proteins upon immunofixation, a chemiluminescent technique was employed to study the prevalence of oligoclonal antibodies in these sera (chapter 2).

The antibody response in all the reactive sera against the 16kDa antigen was oligoclonal, superimposed on a polyclonal background. Six of the sera showing seroreactivity to 16kDa (above cut-off value) by ELISA demonstrated an oligoclonal response by IEF immunofixation; three sera lacked reactivity under these conditions. Four sera, which were not positive for anti-16kDa antibody by ELISA, demonstrated an oligoclonal response (Table 4.3). A positive correlation was observed between anti-16kDa antibody levels measured by ELISA and prevalence of oligoclonal antibody in the sera analysed by IEF immunofixation ( $r=0.43$ ,  $p=0.024$ ). However, there was no relationship with antibody levels to 38kDa measured by ELISA and oligoclonal response to this antigen. When the antibody responses to these antigens analysed by IEF immunofixation were compared irrespective of quality of the antibody, they correlated positively ( $r=0.54$ ,  $p=0.005$ ). The same observation was made with ELISA. However, when the antibody response to these antigens was compared respective of quality of antibody, no correlation was observed.

There was no relationship between the prevalence of an oligoclonal antibody response against the 16kDa and 38kDa antigens and reactivity to tuberculin skin test (considered  $\geq 10$  mm positive).

16kDa	ELISA +	9/27 (33%)	P	6/9 (66%)
			O	6/9 (66%)
	ELISA -	18/27 (66%)	P	4/18 (22%)
			O	4/18 (22%)
38kDa	ELISA +	7/27 (26%)	P	4/7 (57%)
			O	1/7 (14%)
	ELISA -	20/27 (74%)	P	4/20 (20%)
			O	3/20 (15%)

**Table 4.3: Prevalence of B cell clones amongst sera from geriatric subjects**

P: Polyclonal response; O: Oligoclonal response

#### 4.2.4 Discussion

##### 4.2.4.1 Prevaience of an oligoclonal response in TBM patients

The simple immunoblotting assay employed in this study is as informative as the ELISA, and indicates whether an antibody response is present against a particular antigen.

In the CSF of a healthy individual, there are only up to four white cells per ml present and inflammatory proteins and immunoglobulins are represented at approximately 1:400 of their serum concentration. Therefore, the sample concentration was used in the ratio of 1: 400 of serum:CSF.

Screening of CSF and matched sera from one TBM patient, and three multiple sclerosis patients using a simple immunoblot assay revealed specific antibodies against the 16kDa and 38kDa antigens in the TBM patient, not in any of the multiple sclerosis patients. Antibody response was observed in both serum and CSF, indicating passive transfer of IgG between blood and CSF through an inflamed or damaged blood brain barrier (BBB). However, increased intensity of staining in the CSF compared to serum indicated intrathecal synthesis. MTB sonicate antigen, a mixture of crude poorly-defined mycobacterial antigens, could not discriminate the TBM patient from multiple sclerosis patients, indicating cross-reactivity.

IEF has optimal resolving capacity for determining the electrophoretic pattern of immunoglobulins (Igs) in undiluted CSF (Walker, *et al.*, 1983a). Its high resolving power improves the resolution of single bands seen by other electrophoretic techniques, in the gamma region of CSF of patients with central nervous system disorders. Link (1967) demonstrated that fractionation/resolution of the gamma region results in IgG bands of restricted termed heterogeneity, "oligoclonal" (Link, 1967). Oligoclonal bands restricted to the CSF are the only reliable indicators of intrathecal IgG and are practically always associated with inflammatory disease of the central nervous system (CNS) (Thompson and Keir, 1990). Thus, if bands are present in the gamma region of CSF, which are absent in the homologous serum, then there is quantitative evidence for intrathecal synthesis of gammaglobulin. Therefore, the concept is that CSF gammaglobulin in normal

patients is all derived from the serum, whereas the elevated CSF gammaglobulin found in inflammatory disease of the central nervous system is synthesised locally.

In the present study, elevated anti-38kDa and 16kDa oligoclonal antibody levels in the CSF compared to matched serum samples was detected in the TBM patient, indicating local expansion of specific clones. Interestingly, a very low polyclonal response for these antigens was observed in the CSF of this TBM patient. This suggests a predominant specific expansion of clones against these antigens. Previous studies have demonstrated intrathecal oligoclonal IgG bands superimposed on a polyclonal background to MTB sonicate (Kinnman, *et al.*, 1981, Sindic, *et al.*, 1990). The strong polyclonal response observed in the earlier studies might have been due to the cross-reactive nature of the MTB extract they used. Furthermore, a stronger polyclonal response makes it difficult to visualise oligoclonal bands. This study has shown that employing species-specific antigen can reduce the cross reactivity and thereby reduce the polyclonal background.

Previous work has shown that oligoclonal bands were produced at the same pI points for MTB, *M.vaccae* and *M.avium* sonicates (Davies, 1988). Based on this finding, it was concluded that antibodies were produced against common antigens (group (i) antigens), rather than species-specific antigens. However, the present study has now shown that oligoclonal antibodies against species-specific antigens are produced within the CSF during the course of the disease.

Elevation of levels of antibody in CSF of TBM patients to 16-, 19-, and 38-kDa antigens detected by ELISA has been reported previously (Chandramuki, *et al.*, 1989). Species-specific antigens of the *M. tuberculosis* complex are theoretically attractive immunodiagnostic reagents. The high sensitivity of the serological tests using the purified 38-kDa antigen in pulmonary tuberculosis patients, suggests its diagnostic value (Espitia, *et al.*, 1989, Wilkinson, *et al.*, 1997). The presence of high-density oligoclonal bands in the undiluted CSF in the TBM patient in the present study accords well with these previous observations. Even though anti-16kDa antibody has been demonstrated by ELISA in TBM patients in various studies, the intensity and number of oligoclonal bands against this same antigen observed in this study was very low compared to the anti-38kDa oligoclonal

response. Immunological data from previous studies indicate that there was a poor antibody response to these antigens in a proportion of patients (Lyashchenko, *et al.*, 1998, Gennaro, 2000). Therefore, use of these two antigens in IEF as a serodiagnostic reagent needs further investigation with a larger number of samples.

Further CSF specimens with matched sera from TBM patients could not be obtained for additional evaluation of this method. Therefore, 28 CSF specimens without matched sera from TBM patients were studied to evaluate the diagnostic value of this method. In this study, in addition to bacteriologically proven TBM, the gold standard for diagnosis, response to antituberculosis therapy was also considered as an alternative gold standard for diagnosis of TBM.

While antibodies binding to 38kDa antigen were detected by immunoblotting in 44% (12/27) of all subjects with TBM, immunofixation after focusing revealed oligoclonal antibodies against this antigen in 70% (19/27) of subjects. This is because IEF allows excellent resolution of proteins, thereby even a trace amount of antibody in the specimen can be fractionated and subsequent immunofixation against the antigen results in efficient binding of antibodies to the antigen. Only four out of seven (57%) culture proven CSFs and fifteen out of twenty CSFs from TBM cases, that responded to therapy revealed an oligoclonal antibody response against the 38kDa antigen. This may be due to the mopping up of the antibodies by the mycobacterial antigens, which are being released from MTB (Chandramuki, *et al.*, 1985).

Immune complex assays to detect anti-mycobacterial immune complexes have been shown to be superior in the detection of anti-mycobacterial antibody (Miorner, *et al.*, 1995, Patil, *et al.*, 1996), indicating a reduction in freely available antibodies and an increase in antigen antibody complexes. This means free antibodies will not be available in a proportion of TBM patients, which results in a false negative. Other studies have also shown that detection of mycobacterial antigen in CSF of TBM patients has increased sensitivity compared to detection of anti-mycobacterial antibodies (Radhakrishnan, *et al.*, 1990, Krishnan and Mathai, 1991).

The polyclonal response to the 38kDa antigen found in three of the negative controls highlights the need for the measurement of antibody titre in the matched serum samples from these patients. It should be noted none of these negative controls revealed any oligoclonal response for this antigen, suggesting a lack of specific clonal expansion. Therefore, the polyclonal antibodies could have been passively transferred from the blood to CSF.

Only six out of twenty seven (22%) of CSF samples from TBM patients reacted to the 16kDa antigen. All of these samples only revealed a polyclonal response. Only one of the negative controls from a British patient revealed a polyclonal response against the 16kDa antigen. It should be noted that if the intensity of staining of the oligoclonal bands are low, their presence could have been masked by the intensive staining of the polyclonal bands. Chandramuki and colleagues reported a sensitivity of 61% for a diagnostic method, which detected IgG antibody titre against 14kDa (16kDa) in the CSF of TBM patients using ELISA (Chandramuki, *et al.*, 1989). It must be remembered that the sensitivity of any immunological assay is related to the extent and chronicity of the disease, as various antigens are expressed during different stages of the infection.

The present study suggests that analysing the quality of mycobacterial antigen-specific levels of antibody in matched serum and CSF would be valuable in the diagnosis of tuberculous meningitis. Detection of IgM, which is much less diffusible than IgG antibody would also increase the specificity of this technique. Furthermore, MTB-specific antigens are a more reliable alternative to the crude extracts of MTB. The only disadvantage of the IEF technique is its tendency to subdivide even normal polyclonal IgG into bands. Therefore, to assess IEF separation more reliable, the kappa, and lambda pattern of these oligoclonal immunoglobulins should be characterised (Walker, *et al.*, 1983b). In addition, detection of IgM antibody titres against 16kDa may improve the diagnostic value of IEF, as this is the first immunoglobulin to appear after initial infection.

#### 4.2.4.2 Prevalence of oligoclonal response in geriatric subjects

It has been established that the oligoclonal response to MTB-specific antigens within the central nervous system represents a feature of MTB-associated clinically relevant conditions (Kinnman, *et al.*, 1981, Sindic, *et al.*, 1990). Persistence of an intrathecal oligoclonal response has been demonstrated in the CSF, even though this was collected 69 months after the onset of disease in TBM patients who had been considered bacteriologically completely cured, but had suffered from major sequelae. Interestingly, no intrathecal oligoclonal antibody was found in those who completely recovered (Sindic, *et al.*, 1990). Various reasons were proposed for this observation, but the possibility of persistence of viable bacilli even after successful treatment was not then considered. A study on Italian military students revealed the prevalence of anti-*H.pylori* oligoclonal antibody in the sera of asymptomatic subjects (Biselli, *et al.*, 1999). During the ten-month follow-up period, anti-*H.pylori* oligoclonal antibody persisted in the subjects, and it has been suggested that chronic infection is associated with prevalence of oligoclonal antibody.

In the present study, among these sero-reactive to the 16kDa antigen, detected either by ELISA or by IEF, 77% (10/13) demonstrated oligoclonal antibodies against this antigen. Only 30% (4/13) of the sera reactive to the 38kDa antigen demonstrated an oligoclonal response. There was no relationship between oligoclonal responses to these antigens, indicating the prevalence of specific B cell expansion only to the 16kDa antigen in this elderly group. This is in contrast to the 38kDa antigen, to which a high prevalence of specific B cell expansion was found in active disease. This study has demonstrated a high level of oligoclonal response to the 16kDa MTB antigen in the sera of latently infected people who were showing high seroreactivity to this antigen by ELISA and a low level of oligoclonal response to this antigen in active disease. Therefore, as hypothesised, anti-16kDa antibody may be a marker for latent infection, but only the evaluation of the presence of an oligoclonal response can reliably diagnose latent infection. Quantitative measurement of IgG in the sera is of no value in diagnosis of latent infection. The only setback in the present study is that the technique has not been evaluated on sera collected from people who had had no

known contact with tuberculosis. Neither has, this study evaluated the sensitivity and specificity of this method. It also needs to be evaluated in a large population with known BCG status, to study the efficiency of this method to discriminate BCG-vaccinated subjects from latently infected subjects.

As there is no gold standard for diagnosis of latent tuberculosis infection, assessment of accuracy of a new assay is difficult. A recently proposed ESAT-6 enzyme-linked immunospot (ELISPOT) assay for diagnosis of latent infection yielded promising results (Lalvani, *et al.*, 2001a, Lalvani, *et al.*, 2001b, Lalvani, *et al.*, 2001c). Therefore, the method described in this study should be compared with the ELISPOT method to evaluate its accuracy.

## **Chapter 5**

### **5. Polymerase Chain Reaction in the diagnosis of latent tuberculosis infection**

#### **5.1 Polymerase chain reaction of sputum and nasal swabs of institutionalised elderly persons for mycobacterial DNA**

##### **5.1.1 Introduction**

It is a general belief that latently infected people do not shed the bacilli and transmit the disease until they develop reactivation of the disease. Stead and colleagues have reported an increase in the proportion of tuberculin conversion among elderly nursing home residents even in homes in which no source case had been identified (Stead, *et al.*, 1985). One can argue that reaction to tuberculin is not specific; therefore, tuberculin conversion does not necessarily mean that the person is infected with MTB. This is not rational, as it has been shown that while only 0.02% of non-reactors to tuberculin developed active TB, 3% of reactors developed active TB (Stead and To, 1987). MTB DNA has been detected in the sputa of treated elderly leprosy patients using the IS6110 primers that detect MTB DNA with a detection limit of fewer than 10 bacilli (Rafi and Feval, 1995). These observations raise the question whether latently infected people without reactivation may shed bacilli and transmit tuberculosis to others.

Therefore, in the present study, sputum samples from the elderly whose sero-reactivity to MTB-specific antigens is described in Chapter 4, were screened for the presence of MTB by acid-fast staining, culture and polymerase chain reaction in their sputum. In addition, nasal swabs from these elderly were also subjected to PCR analysis with the view that if transmission of tubercle bacilli occurs MTB DNA would be present in the mucosa of the upper respiratory tract. The nose acts as an air filter, and so inhaled bacilli may concentrate there and be detectable.

Nasal swabs were also screened for *M. leprae* DNA to measure the sub-clinical leprosy infection in this population. Previous studies have shown the presence of

*M. leprae* DNA in the nasal swab specimens of occupational contacts and people from endemic area (de Wit, *et al.*, 1993).

### **5.1.2 Method and materials**

#### **5.1.2.1 Sputum samples**

All together 29 sputum samples were obtained from twenty institutionalised geriatric subjects who were included in the serology study described in chapter 4. Repeat samples were obtained from nine subjects, one year after the collection of the first sample. Sputum samples from those who were able to produce one were collected into a wide-mouth-screw-capped sterile container. Collected samples were stored for not more than four days at 4°C, transported to the study location at room temperature, and processed within 48 hours. However, few sputum samples that had to be stored for longer, were kept at -20°C. Sputum samples from geriatrics were analysed on three different occasions. All the repeated samples were analysed on one occasion. Sputum samples were also obtained from four individuals (average age 27) working in the institution from where the geriatrics subjects were recruited for this study. Four culture-positive sputum samples and five culture-negative sputum samples were also tested, using the same extraction and amplification methods used to analyse the test samples, as positive and negative controls, respectively.

#### **5.1.2.2 Nasal swabs**

Nasal swabs were taken from ten geriatric subjects. The sterile calcium alginate swabs were dipped in sterile saline before use and nasal swabs were stored at -20°C and transported to the study location in a cold box with ice. At the destination, they were kept at -20°C until processed for PCR. Swabs dipped in sputum specimens from culture positive patients were used as positive controls for the nasal swabs PCR.

### **5.1.2.3 Direct microscopy and culture**

All the test sputum samples were screened for the prevalence of MTB by culture and stained by the Ziehl-Neelsen and Auramine methods.

### **5.1.2.4 PCR**

Sputum samples were screened for mycobacterial protein genes and a MTB complex-specific region of the insertion sequence IS6110. DNA was extracted from sputum samples using guanidiumthiocyanate (GuSCN) and silica suspension as described in chapter 2. In addition to this extraction method, a commercially available extraction kit (PureGene) was used to extract DNA from nasal swabs. Three sets of oligonucleotide primers were used to amplify target DNA from the genes encoding the 65kDa and 19kDa mycobacterial antigen genes, and IS6110. In addition to the above-mentioned primers, nasal swabs were also screened for the *M.leprae* repetitive element RLEP. Extraction procedures, primer sequences, and PCR procedures are described in chapter 2 under section 2.7. As with all PCRs, a known positive control was amplified to ascertain the reagent and the procedure efficiency and negative DNA extraction and PCR controls were included.

Negative samples were screened for possible inhibition of the PCR by using a set of primers that amplify human mitochondrial DNA (mtDNA) (primer sequence and PCR procedure are given in chapter 2).

## **5.1.3 Results**

None of the sputum samples obtained from geriatric subjects and hospital workers were positive for *M. tuberculosis* either by culture or by staining. Cultures were incubated for a period of 18 weeks. In contrast, PCR detected the presence of the 92bp DNA fragment specific for the MTB complex in eleven (55%) sputum samples from the elderly. Out of these eleven samples, five were also positive for the 131bp DNA fragment of the 19kDa antigen gene. None of the samples was positive for the 439bp PCR product of the 65kDa antigen gene, which is found in all mycobacteria. Good concordance was found with the repeated samples,

except for one sample from a geriatric subject, whose initial sputum sample was positive for both 92bp and 131bp DNA fragments. All five sputum samples that were negative for MTB DNA initially were negative when repeated. All positive and negative controls were satisfactory. No PCR inhibitors were detected in the PCR negative specimens. It was noted that even though silica was used in the DNA extraction procedure to adsorb DNA, no amplified product was obtained from DNA eluted from the silica deposit. In contrast, a positive amplification was always obtained from the DNA extracted from the silica supernatant.

The results of PCR, the tuberculin skin test and prevalence of oligoclonal antibodies against 16kDa and 38kDa recombinant MTB-specific antigens are given in table 5.1. Of eleven elderly persons who were positive for MTB DNA in their sputum, only two showed an oligoclonal response against the 16kDa antigen of MTB.

None of the DNA targets tested in this study was detected in any of the nasal swab samples. In contrast, all of these samples were positive for human mitochondrial DNA.

Institutionalised elderly subjects	Skin test (mm)	PCR		Oligoclonal response	
		IS6110 (92bp)	19kDa (131bp)	16kDa	38kDa
1	0	+	+	+	-
2	2	+	+	NT	NT
3	14	-	-	-	-
4	10	+	+	-	-
5	5	-	-	NT	NT
6	0	-	-	NT	NT
7	12	-	-	-	-
8	15	+	-	NT	NT
9	0	+	+	NT	NT
10	20	+	+	-	-
11	12	-	-	-	-
12	10	+	-	-	-
13	12	-	-	-	-
14	12	-	-	-	-
15	10	+	-	+	+
16	12	+	-	-	+
17	15	+	-	-	-
18	0	-	-	+	-
19	0	+	+	-	-
20	0	-	-	-	-

**Table 5.1: Over all result of PCR and prevalence of anti-16kDa and 38kDa oligoclonal antibody in association with tuberculin skin test in individual geriatric subjects**

NT: Not tested

#### 5.1.4 Discussion

Several studies have described the increased sensitivity of molecular approaches to the diagnosis of tuberculosis over conventional diagnostic methods (Rafi and Feval, 1995, Li, *et al.*, 2000, Pai, *et al.*, 2000, van der Zanden, *et al.*, 2001, Ashino, *et al.*, 2002). The multicopy IS6110 gene has been the most commonly utilised target in in-house assays for the molecular detection of the MTB complex (Kaul, 2001). In the present study, the region between 762 and 884 of the IS6110 element was utilised as the target. Even though the DNA sequence of certain regions of this particular insertion sequence were found to have homology with non-tuberculous mycobacteria, the target utilised in this study has been proved to be specific for the MTB complex (Eisenach, *et al.*, 1991, Hellyer, *et al.*, 1996).

IS6110 copy number per strain can range from none to about 25 copies. The proportion of MTB that lack IS6110 gene differs significantly by geographical area (Van Soolingen, 2001). For example, MTB isolates that were obtained from sputa of Vietnamese and Indian (Madras) TB patients had either no copy or only a single copy of IS6110 (Yuen, *et al.*, 1993, Sahadevan, *et al.*, 1995). Therefore, detection of IS6110 targets may result in false negatives in those strains that lack IS6110. Thus, a more useful approach in such a situation would be amplification of a common mycobacterial target, followed by a specific target.

Therefore, in the present study, a 439bp fragment of the gene encoding 65kDa protein was amplified in order to reduce the chance of false negatives, in case the strains lacked the IS6110 element. Using these primers Telenti and colleagues showed amplification of DNA from 43 different reference strains of mycobacteria and 290 clinical isolates of mycobacteria, suggesting that it was a common mycobacterial target (Telenti, *et al.*, 1993). Unfortunately, none of the samples in the present study was positive for the 65kDa target, including the culture positive sputum samples. However, amplification was observed in the positive control, which was of DNA extracted from a MTB culture. Various factors may have hampered amplification of this target, single copy of the target gene, large target size, inhibitors in the clinical samples and high GC content of the target DNA.

Compared to multicopy targets, amplification of single copy targets is difficult especially in clinical samples where the quantity of DNA would be very low. In addition, quality of DNA is a problem in clinical samples as such DNA is fragmented and damaged due to various constraints on the bacilli *in vivo*. Therefore, amplification of large targets such as the 439bp is difficult in comparison with small fragments such as 92bp.

A previous study employed a 383bp DNA fragment from this gene (encoding 65kDa protein) as a target to detect mycobacterial DNA extracted from sputum specimens. It was reported that the 383bp DNA fragment was amplified only when dimethylsulphoxide (DMSO) was added to the reaction mixture (Mustafa, *et al.*, 1995). Certain organic solvents have been reported to enhance PCR

amplification, particularly for hard-to-amplify high-GC templates (Chakrabarti and Schutt, 2001, Chakrabarti and Schutt, 2002). Since the common mycobacterial target template utilised in this study was large and rich in GC content, the addition of an organic solvent may have enhanced amplification.

In addition to the *IS6110* target, a second 131bp target from the gene encoding the 19kDa MTB protein has also been amplified to confirm the prevalence of MTB DNA in the sputa of elderly subjects. This is a single copy target. This target was detected only in 54% (6/11) of these samples, which were positive for *IS6110*. None of the samples negative for *IS6110* samples was positive for the 131bp target, suggest the accuracy of the PCR. This target has also been found in *M. scrofulaceum*, *M. avium* and *M. intercellulare* (Booth, *et al.*, 1993, Mustafa, *et al.*, 1995). Nevertheless, since the *IS6110* target was present in all the samples that were positive for the 19kDa target, it was concluded that the mycobacterial DNA detected in the sputum specimens was from strains belonging to the MTB complex.

A general problem associated with PCR in the diagnosis of tuberculosis is the presence of PCR inhibitory substances in the clinical samples (Sudre, *et al.*, 1992, Forbes and Hicks, 1996) as well as inhibitors introduced by the DNA extraction procedure itself (Suffys, *et al.*, 2001), which result in false negatives. During the last decade, several methods have been published including a very promising method using GuSCN, which was used for the purification of nucleic acids from the sputum samples in the present study. However, a recent study has revealed that an inhibitor of DNA-modifying enzymes can be introduced into the samples during the extraction procedure and co-eluted with nucleic acids during the last step of DNA elution from the silica deposit (Boom, *et al.*, 1999). Furthermore, this investigation revealed that the inhibitor originated from the size-fractionated silica particle suspension and appeared to be tightly bound to the silica particles. This may be one of the factors responsible for the failure of amplification of MTB DNA in DNA extracts eluted from silica, whereas positive amplification was obtained with the silica supernatant in the present study. Boom and colleagues found from their study that addition of alpha-casein to L6 buffer would prevent silica particles from binding to inhibitors (Boom, *et al.*, 1999). If

time had permitted, efficiency of lysis buffer containing alpha-casein could have been evaluated.

Another possible factor that may have interfered with DNA purification was pH of the silica/GuSCN buffer. Binding of DNA to silica takes place at very high acidic pH (pH=2.0) (Boom, *et al.*, 1990). The processed sputum samples used for DNA extraction in the present study were highly alkali as they were initially liquefied with sodium hydroxide. Therefore, high pH of the mixture may have prevented DNA from binding to silica and subsequently resulting in a reduction in the DNA yield.

In the present study, even with silica supernatant, positive amplification was obtained only when bovine serum albumin (BSA) was added to the PCR mixture. Albumin is a known scavenger of PCR inhibitors (Forbes and Hicks, 1996). To identify inhibitors, internal control DNA has been amplified either using the same primers that amplify the target DNA fragment, or using primers that amplify a target expected to be present in large number in that particular DNA extract within the same reaction tube (Mustafa, *et al.*, 1995). Alternatively, the PCR can be repeated for amplification of control DNA in all the samples that are PCR negative for MTB. In the present study, human mitochondrial DNA (mtDNA) was amplified to rule out false negatives due to the presence of inhibitors.

The prevalence of MTB DNA in the sputum samples of the elderly is very intriguing. All the precautions were taken including inclusion of negative controls in DNA extraction and in amplification to avoid false positive results. Except for one sample, good concordance was found between the results obtained with the initial sample and the repeated samples, supporting the study finding. However, a disappointment in this method is its inability to distinguish between viable and non-viable organisms. MTB DNA has been detected in the sputa of patients up to 2.5 years after completion of directly observed therapy (DOT) (Kaul, 2001). Therefore, it is not known whether the geriatric subjects in this study shed viable bacilli and were able to transmit the disease. Even though cultures performed on these sputum samples were negative, the possibility of bacillary viability cannot be excluded as a somewhat similar situation was found in the "sterile state"

mouse of the Cornell model. Recently Pai and colleagues (Pai, *et al.*, 2000) have revealed that in fact the MTB DNA detected in the "sterile state" mouse by Mitichison and colleagues (de Wit, *et al.*, 1995) was from viable bacilli. Measurement of sputum MTB mRNA for 85B antigen has been shown to be effective to assess the response to chemotherapy, as an association was found in the decline in the levels of mRNA for the 85B antigen and viable count of MTB in sequential sputum samples collected from smear-positive pulmonary tuberculosis patients (DesJardin, *et al.*, 1999). Therefore, evaluation of the prevalence of mRNA for the 85B antigen of MTB should reveal the state of the bacilli in the sputum samples of the elderly.

If the DNA detected in this study represents viable bacilli in the sputum, necessary steps need to be taken to treat these elderly persons with prophylactic anti-tubercular drugs. Having said this, none of the individuals had been clinically diagnosed, as suffering from pulmonary tuberculosis and neither were they when clinically assessed after these results were reported. In addition, none of the nasal swabs taken from the elderly were positive for mycobacterial DNA. It was expected that as tuberculosis is transmitted through inhalation of droplet nuclei, some bacilli would be present in the nasal mucosa. There is only one study that has described the prevalence of MTB DNA in the nasal mucosa of TB patients, but the rate of prevalence was fewer than expected (Warndorf, *et al.*, 1996). The same study found that out of 13 close contacts to TB patients, only one was positive for MTB DNA in his nasal mucosa. In contrast, the prevalence of *M. leprae* DNA in the nasal mucosa of leprosy patients, close contacts and even in people living in leprosy endemic areas has been reported (de Wit, *et al.*, 1993). None of the elderly screened in this study were positive for *M. leprae* DNA in their nasal mucosa, even though human mtDNA was amplified in the same DNA extract. However, it was reported that  $10^4$  times more human mtDNA needs to be recovered to detect *H.pylori* DNA from museum samples, assuming similar rates of degradation for the DNA of both species (Barnes, *et al.*, 2000) and a similar disproportion between human and *M. leprae* DNA may occur in the present study.

There is no reason to reject the hypothesis that the elderly who are latently infected may shed viable bacilli. However, the prevalence of MTB DNA in the sputum of the elderly, either from viable or non-viable bacilli, explains the observation made by Stead (Stead, *et al.*, 1985); of conversion to tuberculin positivity even in the absence of an index case in the vicinity. In the present study, no correlation was found between prevalence of MTB and skin reaction to tuberculin when the cut-off for reaction size was considered as 10 mm or more. However, four of the elderly whose sputa were positive for MTB DNA elicited a reaction size of 0 or 2 mm. All the other seven elderly (64%) who were found to have MTB DNA in the sputa elicited a reaction size of 10 mm or more. In comparison, of the PCR negative individuals, 55% (5/9) elicited 10 mm or greater response to tuberculin. The result to date come only from a small number of subjects, and it would be useful to expand this investigation as an adjunct to diagnosis of latent tuberculosis infection.

If the MTB DNA detected in the sputa of the elderly in the present study is from viable bacilli, this suggests that during latent infection the bacilli are metabolising and slowly replicating. This view is further supported by the observation of long term protection conferred by isoniazid in latently infected immunocompetent persons (Grange, 2001b). Based on these observations it is tempting to hypothesise that the immune system of those latently infected should be continuously stimulated by the persisting and metabolising bacilli. If it is the case, there should be an antibody response against MTB-specific antigens. However, in the present study, no association was found between the prevalence of MTB DNA in the sputum and any oligoclonal response to the MTB-specific antigens. This may have occurred due to factors related to aging. Senescent B cells lose their ability to function and respond normally. It has been noted that the B cells isolated from the elderly produced less antibody than B cells from younger subjects (Haeney, 1994). On the other hand, failure to find a positive correlation between prevalence of MTB DNA in the sputa and the antibody response may be due to the reciprocal relationship between antibody production and cell mediated immunity, well known in human tuberculosis. Wilkinson and colleagues found the highest T-cell response (IFN-gamma) and lowest antibody level to 16kDa antigen in healthy BCG-sensitised subjects. In contrast, the antibody level was high in

patients with extensive disease (Wilkinson, *et al.*, 1998). The elderly persons included in the present study were not suffering from active disease therefore they may have had a pronounced T-cell mediated response, Th1, which is the protective type, rather than a humoral response. Hence, this study population could be used as a model to investigate the relationship between humoral and T cell mediated response in latently infected people.

If transmission from person to person has occurred, a uniform molecular fingerprint should be found of the DNA, isolated from at least the close contacts. Therefore, the DNA isolates were spoligotyped to study transmission pattern in the nursing home setting.

## **5.2 Study of epidemiology of tuberculosis infection by use of molecular fingerprinting among a latently infected geriatric population**

### **5.2.1 Introduction**

Until about two decades ago, the only markers available to study inter-person transmission of TB were drug susceptibility profiles and phage types (Gruft, *et al.*, 1984, Crawford and Bates, 1985). However, in recent years, a large number of DNA fingerprinting methods have become available to type mycobacterial isolates. Among the available typing methods, restriction fragment length polymorphism (RFLP) and spoligotyping are widely used to study the transmission of tuberculosis. RFLP is based on the concept that these patterns reflect the copy numbers and the presence of the *IS6110* element at different sites in the genome of MTB complex strains (Van Soolingen, 2001). Although *IS6110* RFLP typing is the most widely applied typing method, it has several setbacks. For RFLP typing, about two µg of DNA of the MTB strain is needed (Van Soolingen, 2001). This amount of DNA can only be extracted from a large number of bacteria grown from clinical material, suggesting that epidemiological data cannot be obtained from culture negative clinical samples, which is a frequently encountered difficulty in the diagnosis of tuberculosis. Another disadvantage of RFLP typing is that it cannot sufficiently discriminate strains with a low copy number of the *IS6110* element. Furthermore, some strains completely lack the *IS6110* insertion sequence (Goyal, *et al.*, 1997). Spoligotyping performs better than RFLP typing in the above-mentioned contexts. This method is based on the polymorphism and visualisation of the spacer DNA sequences in between the 36bp direct repeats in the genomic DR region of MTB complex strains. The principle of spoligotyping is explained in detail in chapter 2, section 2.8. Spoligotyping can be performed on DNA isolated from clinical samples (Kamerbeek, *et al.*, 1997) and is used as an additional typing method for strains with fewer than 5 copies of *IS6110*. In addition, ten femtograms (10 fg) of chromosomal DNA, an amount corresponding to about two mycobacterial genomes, is the lower limit for the reproducible detection of MTB complex strains (Kamerbeek, *et al.*, 1997). However, it should be noted that a proportion of MTB strains with marked differences in their *IS6110* RFLP patterns exhibit an identical spoligotyping pattern (Van Soolingen, 2001).

Therefore, in the present study, spoligotyping was employed as the DNA fingerprinting method to study whether the geriatric subjects harbouring the MTB DNA are transmitting the bacilli to others. In addition, since the elderly population investigated in the present study have lived during the decades when bovine tuberculosis was prevalent, spoligotyping should allow identification of the individual members of the MTB complex in this population.

### **5.2.2 Methods and materials**

Experimental details are described in Chapter 2. BSA was included in the PCR mix, "Ready. To. Go™" PCR Beads (Amersham Pharmacia biotech) were used to amplify the whole DR locus.

DNA extracted from the following samples were spoligotyped: Sputum samples from elderly (29 samples); four workers from the elderly nursing home; and culture-confirmed four positive controls used in the PCR screening. Negative extraction controls and PCR controls were also spoligotyped.

Spoligotyping patterns were analysed using the appropriate function of Excel, Microsoft Office, and compared with the database obtained from the National Institute of Public Health and the Environment (RIVM), The Netherlands. In the present study, results were described using the octal system (Dale, *et al.*, 2001). Figure 5.1 illustrating an example of an interpretation of spoligotyping pattern in octal format. Briefly, the spoligotyping pattern was read in groups of three: the binary series 000, 001, 010, 011, 100, 101, 110, 111 were read respectively as 0, 1, 2, 3, 4, 5, 6, 7. Following 14 groups of three, the 43<sup>rd</sup> spacer was read on its own, simply as 0 or one.

Original pattern (H37Rv)	
Binary pattern	111111111111111100111111111000011111111
Octal blocks	111 111 111 111 111 111 100 111 111 111 110 000 111 111 1
Octal code	7 7 7 7 7 7 4 7 7 7 6 0 7 7 1
Written as	7 7 7 7 7 7 4 7 7 7 6 0 7 7 1

**Figure 5.1: Conversion of original spoligotyping pattern of *Mycobacterium tuberculosis* strain H37Rv, shown diagrammatically to octal code**

Adapted from (Dale, *et al.*, 2001)

### 5.2.3 Results

Each DNA extract was subjected to amplification using neat, 1/5, 1/10 and 1/20 dilutions of samples. Truncated or no spoligotyping patterns were obtained using undiluted DNA extracts. Complete spoligotyping patterns were obtained either using 1/5 or 1/10 diluted DNA extracts. Low intensity spots were observed in some of the spoligotyping patterns. In some instances combination of 1/5 and 1/10 diluted DNA spoligotyping patterns were used to get the complete pattern. Spoligotyping was done twice on each sample, and spoligotype patterns were consistent.

MTB complex DNA was detected in eleven of elderly persons, and in all four positive controls (Table 5.2). No spoligotyping patterns were observed in the negative PCR controls, DNA extraction controls, or the nursing home workers.

Subject	Spoligo pattern	PCR IS6110
<b>H37Rv</b>	<b>777777747760771</b>	
<b>P3</b>	<b>676773777777600</b>	
1	775057670600771	+
2	765777652020771	+
3	777777777720371	+
4	777640024020771	+
5	776761177762571	+
6	370217601760771	+
7	777374377760771	+
8	02000000403711	+
9	4000400000000000	+
10	777757647760471	+
11	377077700000771	+
12	77777767360400	-
13	77777777701771	-
14	77777777723771	+
15	77777777762771	+
16	311160013560171	+
17	375401701740130	+

**Table 5.2: Spoligo patterns obtained from DNA from sputum samples.**

*M. tuberculosis* H37Rv and *M. bovis* BCG, P3, generally do not hybridise with spacers 33 to 36 and 39-43, respectively. Subjects, 1-13 elderly persons; 14-17 positive controls.

Two sputum samples that were negative for PCR revealed spoligotype patterns on fingerprinting. Comparison of spoligotyping pattern of subject 12 with the international database of spoligotyping patterns at the National Institute of Public Health and the Environment (RIVM), Netherlands revealed a similar pattern to the spoligotyping pattern of *M. bovis* isolates. An identical spoligotyping pattern to subject 3 has been found in MTB isolates from different geographical locations including Spain. Spoligotyping patterns of samples from all other subjects, except the sample from subject 9, were almost identical to the spoligotype patterns previously described for *M. tuberculosis* isolates. A spoligotype pattern, similar to sample 8 has also been found in *M. microti* isolates from animals. However, an almost identical pattern to *M. microti* animal isolates has been found in *M.*

*tuberculosis* strains isolated from patients from Argentina and Cuba. The sample from subject 9 hybridised only with spacers 1 and 13.

The Spoligotype pattern of original samples and samples obtained after one year is shown in table 5.3.

Subject	Spoligo type pattern
3	A: 77777777720371 B: 777776774760571
5	A: 776761177762571 B: 420000100410431
6	A: 370217601760771 B: 021502000000400
11	A: 377077700000771 B: 016163774720771

**Table 5.3: Octal code representation of results obtained on serial sputum samples from elderly persons**

A: Initial sample; B: Repeat sample

The repeated sample from subject 3 failed to react with four spacers that were present in the original sample. All other three repeated samples revealed a highly different pattern from the original spoligotype patterns.

#### 5.2.4 Discussion

The use of molecular epidemiology to study transmission of MTB is based on the fact that strains recently transmitted from a common source exhibit the same fingerprint. The term 'cluster' is used to indicate MTB isolates with identical or highly similar DNA fingerprints. Analysis of spoligotype patterns of the DNA isolated from the elderly persons has revealed no clustering, suggesting absence of transmission of bacilli within this population. Furthermore, it indicates that the bacilli found in the sputum were endogenous in origin. This observation raises

many questions regarding transmission. Firstly, if bacilli were shed in the sputum why has it not been transmitted from person to person? Secondly, if the subjects under investigation were not actively infected, then why do they shed bacillary DNA? Do the bacilli multiply in these people at a very low rate?

This is the first study of the examination of sputum samples from elderly people for MTB DNA. Another study that examined the sputum samples from treated leprosy patients also showed the presence of MTB DNA in a higher proportion of elderly persons compared to younger people (Rafi and Feval, 1995). It is not known whether healthy young people with compatible and stable abnormal X-rays (classified as inactive TB) also shed bacilli in their sputum.

Morphological changes of the organs and tissues in the elderly may be proposed as one reason for the shedding of bacilli in sputum. Unfortunately, chest X-ray of the elderly population of this study could not be performed because of practical difficulties. However, it is well known that aging changes the lung morphology and biochemistry, which in turn cause changes in lung mechanics and gas exchange. Since these people were not suffering from active TB, the bacilli in the sputum may be in an inactive state, therefore not able to cause infection in a new host. A case study on an 81-year-old women, who had a history of chronic cough, sinusitis, laryngitis, and chronic otitis media disclosed the presence of a single bacillus (only one colony in culture) in the serial sputum samples collected at two month intervals (van der Zanden, *et al.*, 2001). This indicates that the number of bacilli in the sputum is very low in those suffering from a sub-clinical infection, and therefore the infectious dose may not be sufficient to cause a new infection in a healthy person. It is interesting to postulate that such a challenge might be sufficient to infect an immunodeficient person.

Spoligotyping has been used successfully to simultaneously detect and type MTB in clinical materials such as sputum, microscopic preparations stained for the detection of acid-fast bacilli and paraffin-embedded tissues (Kamerbeek, *et al.*, 1997). In the present study, it detected MTB DNA in two samples that were negative by conventional PCR using primers based on *IS6110* element. Spoligotyping may be superior to conventional PCR based on *IS6110* as the target DNA is smaller, only about 55bp and it can identify MTB isolates that lack

IS6110. Smaller fragments targeted in spoligotyping increases its sensitivity especially with DNA extracts obtained from clinical samples. It is difficult to obtain good quality and enough quantity of DNA from these sources for RLFP typing.

Incidentally, one of the samples negative for MTB DNA by conventional PCR turned out to be *M. bovis*, which has only one or very few copies of IS6110 element at a fixed genomic position (van Soolingen, *et al.*, 1994). Identification of *M. bovis* in the elderly population further supports the endogenous origin of the bacilli detected in this study. Human tuberculosis due to *M. bovis* is now very rare in developed nations (Grange, 2001a). It was common before the bovine tuberculosis eradication programme in 1960. The presence of *M. bovis* DNA in an elderly person suggests that infection was acquired before 1960 and persisted as a latent infection.

In the present study, DNA extracted from subject 9 hybridised with only two spacers (1 and 13), however no identical pattern to this spoligotype pattern is in the database held at RIVM. If the number of bacteria present in the clinical sample is low, it can result in an incomplete spoligotype pattern (Kamerbeek, *et al.*, 1997, van der Zanden, *et al.*, 2001). In the present study, complete patterns were observed when diluted DNA extracts were used. Dilution of DNA extracts reduces the amount of PCR inhibitors and at the same time, it diminishes the amount of DNA available for amplification. This is a major setback of spoligotyping directly on clinical samples.

Repeated samples from elderly persons did not produce an identical spoligotype pattern to the one seen with the initial sample. This may be partly due to the PCR inhibitors, quantity, and quality of the MTB DNA in the sputum. Therefore, optimisation of pre-treatment of clinical material prior to spoligotyping is vital.

The failure to obtain an identical pattern might have also occurred due to instability of the DR locus and /or due to multiple strain infection.

Instability of the DR locus may be a reason for the minor change in the repeated sample of the subject 3 where a difference was observed in four spacers. The

stability of *IS6110* plays an important role in the utilisation of fingerprinting in epidemiological studies. If transmission takes place more rapidly than the window for epidemiological investigation, linked cases will be missed. On the other hand, long-term stability of *IS6110* may lead to an overestimation of recent transmission. The stability of the DR locus depends on two factors: homologous recombination between adjacent or distant direct variable repeats and *IS6110* transposition (Groenen, *et al.*, 1993). Controversial evidence exists regarding the stability of *IS6110* element. Some changes in *IS6110* banding patterns have been noted for isolates from cultures collected more than 90 days apart from the same individual (Yeh, *et al.*, 1998). On the other hand, de Boer and colleagues, based on isolates from Dutch patients, estimated that the half-life of *IS6110* RFLP was 3-4 years (de Boer, *et al.*, 1999). Recently Benjamin *et al* reported a single transposition event of *IS6110* in a strain within 2 days (Benjamin, *et al.*, 2001). The specific growth conditions play an important role in the occurrence of transposition of *IS6110* (de Boer, *et al.*, 1999). It has been suggested that adverse conditions may stimulate transpositional events. The bacilli, which lurk in latently infected people are exposed to various adverse conditions, if they persist in granulomas. Therefore, adverse conditions may be one of the reasons for the non-identical spoligotype pattern in the repeated samples.

On the other hand, non-identical pattern observed with the repeated samples might have occurred due to multiple strain infection or due to new exogenous infection. De Boer *et al* showed with single-colony cultures that the MTB population could consist of subpopulations with different *IS6110* RFLP patterns (de Boer, *et al.*, 2000). They observed low-intensity bands in the *IS6110* RLFP of mixed populations. In the present study, low intensity spots were observed in some of the spoligotype patterns, which may indicate infections with multiple MTB strains. An association between occurrence of mixed populations of MTB and increased age has been observed (de Boer, *et al.*, 2000). It is suggested that the occurrence of mixed bacterial populations may be due to gradual changing of the bacterial population in the human body over time or may be due to genetic adaptation of MTB to changes in the environmental conditions during the dormant state (de Boer and van Soolingen, 2001).

In conclusion, the result of this study shows that latently infected people can shed MTB DNA, and possibly viable bacilli, in their sputum, but the infectious dose and/or the virulence of those bacilli is not sufficient to cause new infections as the spoligotyping revealed non-identical patterns among the study population.

## **Chapter 6**

### **6. Cellular localisation of *Mycobacterium tuberculosis* in the lungs of latently infected people**

#### **6.1 Introduction**

The most accepted view of the initial phase of tuberculosis infection is that on inhalation of droplet nuclei, bacilli are taken up by alveolar macrophages (Rook and Bloom, 1994). The subsequent events and the fate of tubercle bacilli taken up by macrophages are reviewed in chapter 1 under section 1.3.1. According to the proposed pathogenesis of tuberculosis, persisting bacilli are assumed to lurk in the macrophages residing within the granulomas. The observation made by Opie and Aronson in the early part of the last century contradicts this assumption (Opie and Aronson, 1927). These investigators reported that fewer than 10% of old lesions contained live bacilli, whereas such organisms could be recovered from macroscopically normal lung tissues in almost 50% of people who died from causes other than tuberculosis. This indicates that during latent infection bacilli lurk not only in granulomas but also in normal lung tissues. This view was further supported by the findings of Balasubramanian who found that while primary lesions occurred anywhere in the lung, progressive cavitary "post-primary" disease almost always occurred in the upper lobes (Balasubramanian, *et al.*, 1994b).

In the present study, conventional PCR and *in situ* PCR were employed to determine whether MTB DNA is indeed present in lung tissues with no specific histopathology and, if so, to seek the cellular localisation of the DNA and, presumably tubercle bacilli.

#### **6.2 Materials and methods**

##### **6.2.1 Patients**

Paraffin-embedded lung tissues of 34 Mexicans obtained at necropsy were selected from archives at the Department of Pathology, Institute of National Nutrition, and at a Children's Hospital, both in Mexico City, Mexico. The tissue sampled was from the hilar region or from the apex near to the pleura. All the subjects were HIV- 1-negative, of unknown tuberculin status.

Six paraffin blocks of lung autopsies performed in the department of Pathology, Haukeland University Hospital, Bergen, Norway were used as negative controls, because national routine tuberculin surveys performed every year have proved that community transmission of tuberculosis has been totally interrupted since the 1950's (Norwegian National Tuberculosis Register, Oslo). Lung sections from six patients with tuberculosis were used as positive controls.

### **6.2.2 Experimental details**

Experimental details are given in Chapter 2. *In situ* PCR and conventional PCR were performed using MTB-specific primers P1 and P2 of the *IS6110* element (Eisenach, *et al.*, 1991). DNA extraction and liquid-phase PCR were done twice on each block on consecutive sections.

Controls for the *in situ* PCR included lung tissues from uninfected mice; lung tissues from people identified as negative for MTB DNA; and sections of lung autopsies from TB patients identified as positive for MTB DNA, to which no Taq DNA polymerase or primers were added. To control for false positives due to DNA repair, duplicates of each test section were subjected to PCR without the MTB-specific primers. The post-amplification PCR mixtures were analysed for diffusion of PCR products out of the cells by agarose gel electrophoresis and ethidium bromide staining.

## **6.3 Results**

Table 6.1 shows the data from the 34 lung tissues of Mexicans tested for MTB DNA by conventional PCR and *in situ* PCR. Summarised results are given in table 6.2. MTB DNA was detected in lung tissues of eight Mexican patients by both conventional and *in situ* PCR. Two were positive only by *in situ* PCR, and four were positive only by conventional PCR. However, results obtained using both methods were concordant in 26 individuals.

Concordance was 100% for the control samples. None of the lung sections from the six Norwegian individuals was positive for MTB DNA by either method.

Conventional PCR detected MTB DNA in all six samples from known TB controls and positive labelling was observed in them by *in situ* PCR.

Histological examination of Mexican samples revealed that despite being devoid of any changes of indicative of TB, tissues were abnormal showing small patches of bronchopneumonia and oedema.

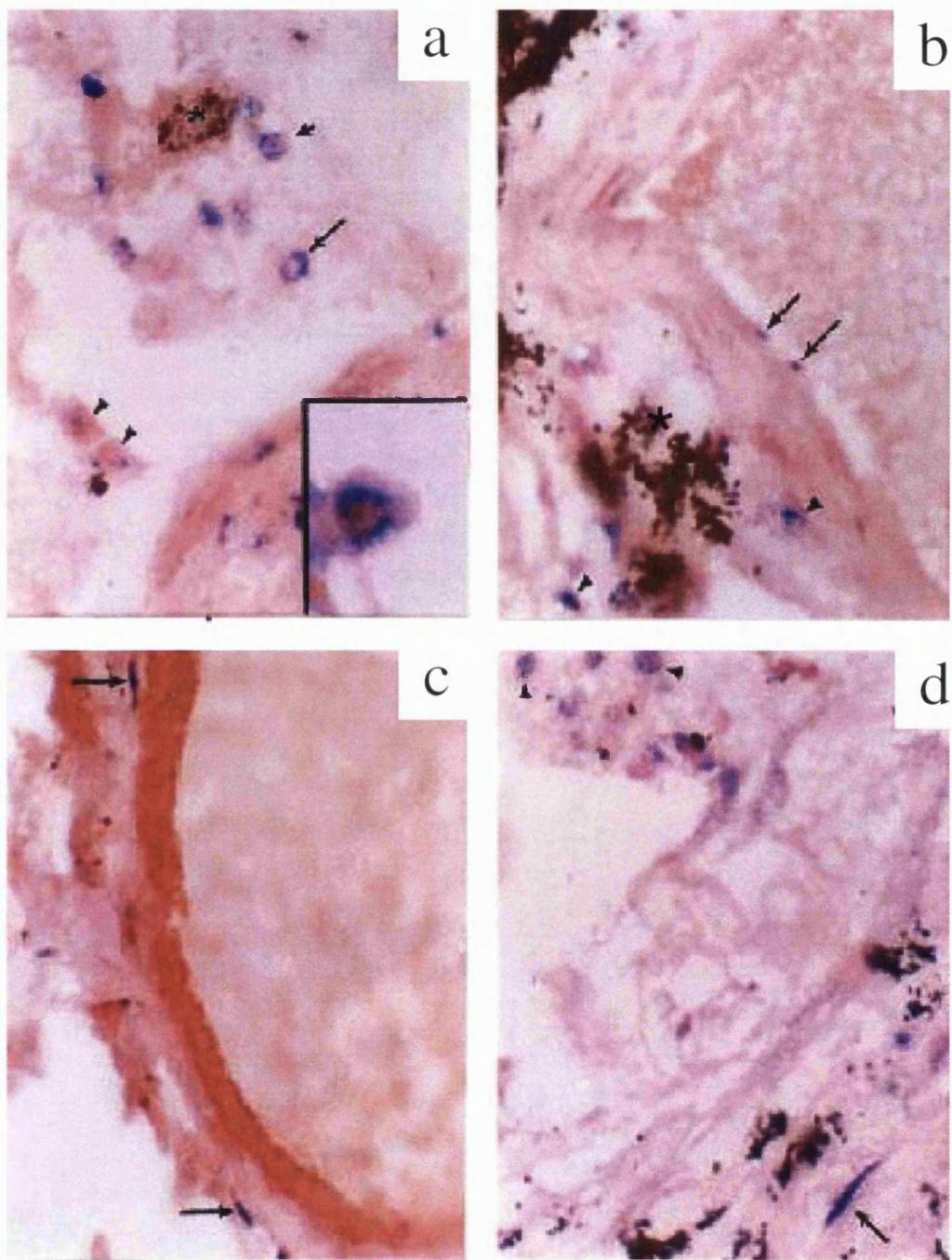
Patients' clinicopathological characters			Presence of MTB DNA	
Age (years)	Sex	Cause of death	<i>In situ</i> PCR	Conventional PCR
Mexican Necropsy				
12	Female	Myeloblastic leukaemia	+	+
17	Female	Alveolar rhabdomyosarcoma	+	+
15	Female	Lymphoblastic leukaemia	+	+
15	Male	Lymphoblastic leukaemia	+	+
52	Female	Myeloma	-	-
56	Female	Myeloid leukaemia	-	-
50	Female	Phaeochromocytoma	-	+
16	Female	Lupus erythromatosus	-	-
23	Female	Lupus erythromatosus	-	-
36	Female	Lupus erythromatosus	-	-
14	Male	Lupus erythromatosus	+	+
22	Female	Goitre	-	-
35	Male	Diabetes mellitus type 1	-	-
45	Female	Diabetes mellitus type 2	-	-
42	Female	Renal failure and diabetes	-	-
37	Female	Starvation	+	+
26	Male	Minimal changes glomerulopathy	-	-
30	Male	Liver cirrhosis	-	-
58	Female	Liver cirrhosis	+	+
57	Female	Liver cirrhosis	-	-
58	Female	Liver cirrhosis	-	+
51	Male	Liver cirrhosis	-	+
46	Male	Liver cirrhosis	-	-
30	Female	Hepatic necrosis	-	-
31	Female	Massive liver necrosis	-	-
51	Male	Acute pancreatitis	-	-
49	Female	Myocardial infection	-	-
55	Female	Sepsis	-	-
48	Male	Sepsis	-	+
6	Male	Cardiac malformation	-	-
12	Male	Cardiac malformation	+	+
13	Male	Cerebral palsy	+	-
58	Female	Stroke	-	-
54	Male	Complication of spinal-cord surgery	+	-
Norwegian necropsy				
38	Male	Hodgkin's disease	-	-
52	Female	Carcinoma of the cervix	-	-
36	Female	Glioblastoma	-	-
49	Female	Melanoma	-	-
45	Female	Multiple sclerosis	-	-
50	Male	Carconomatosis in pericardium	-	-

**Table 6.1: Results of conventional and *in situ* PCR of lung sections from Mexican and Norwegian individuals**

Samples	Age range	Type of PCR		
		In situ PCR	Conventional PCR	Both
Negative controls 6 Norwegians	36-52	0/6	0/6	0/6
	27-40	6/6	6/6	6/6
Positive controls 6 Ethiopian pulmonary TB				
Non-tuberculous patients 34 Mexicans	6-58	10/34	12/34	8/34

**Table 6.2: Over all result of detection of MTB DNA in test samples, positive and negative controls using both PCR methods**

An interesting observation of this study is the cellular localisation of the positive signals. Alveolar and interstitial macrophages were the most common cells with positive MTB DNA labelling (Figure 6.1 (a)). Positive signals were also found in alveolar epithelial cells, but only in pneumocyte type II cells. Some endothelial cells and occasional fibroblasts in venules and intermediate-sized veins were also positive (Figure 6.1 (b) and (c)). Subpleural fibrotic areas showed positivity in macrophages and fibroblasts (Figure 6.1 (d)). Positive cells tended to be grouped together in foci of very variable size, whereas other parts of the same sections were entirely negative. Accumulations of carbon particles were observed in many of the lung tissues of Mexicans and Ethiopians. These were located in the cytoplasm of interstitial or alveolar macrophages, in the fibrous tissue near to the bronchial or vascular walls, or more commonly in the subpleural space (Figure 6.1 (b)).



**Figure 6.1: Representative micrographs of the cellular localisation of mycobacterial DNA revealed by *in situ* PCR in lung tissue from a single donor without tuberculous disease**

A=Alveolar macrophages showing positive (blue) mycobacterial DNA labelling (black arrows). These cells are intermixed with other macrophages containing black phagocytosed carbon material (\*). Positive round cells on the alveolar wall correspond to type II pneumocytes (white arrows and insert). B=Vein endothelial cells (black arrows) and macrophages (arrow heads) surrounding carbon deposits showing mycobacterial DNA labelling. C=Positive fibroblasts (black arrows) located in the advential layer from an intermediate-sized vein. D=A fibroblast (black arrow) and macrophages (arrow heads) in fibrotic subpleural bands show strong mycobacterial DNA labelling. Original magnification X 400 (insert X 100)

#### 6.4 Discussion

An alternative method in general practice to the tuberculin skin test to detect latent tuberculosis infection is a chest radiograph. An abnormal chest radiograph, with typical scars or calcified nodules, is generally considered as an indication for primary tuberculosis (Parrish, *et al.*, 1998). Therefore, persons with such an abnormal chest X-ray are considered as possibly latently infected. This is one of the methods used at the port of entry in many countries to screen immigrants from TB endemic areas. However, the results of the present study and the forgotten study of Opie and Aronson (Opie and Aronson, 1927) question the rationale of this type of screening, as latent infection can be maintained not only in a calcified lesion but can also be maintained in apparently normal lung. Opie and Aronson, and Griffith, transferred lung homogenates from non-tuberculous necropsy samples to guinea pigs, and showed that it is possible to recover live tubercle bacilli from fibrotic or calcified nodules and hilar lymph nodes. However, tissue fragments obtained from the apices or bases of macroscopically normal lung tissue yielded a higher percentage of positive samples than the diseased tissues (Opie and Aronson, 1927, Griffith, 1929). In the present study, using MTB-specific primers from the IS6110 element, which have been shown to have a sensitivity of 95-100% in the diagnosis of tuberculosis in patients who are culture-positive for *M. tuberculosis*, *in situ* PCR and conventional PCR, respectively detected MTB DNA in 29% and 35% of Mexican autopsies of people who died from causes other than TB. There was a 79% overall concordance between the conventional and *in situ* PCR results.

None of the Mexicans included in this study manifested signs and symptoms compatible with TB when they were alive and none of them had shown lesions suggestive of TB in the lungs at necropsy. No histopathological changes indicative of TB were found on microscopic examination. Of interest, a higher rate of PCR positives was found among samples from the paediatric hospital than among samples from adults. This is probably because the children's hospital provides health care for people from a lower socio-economic stratum and it was noted that these children were from the down town area of Mexico City, thus from a population with a high risk of infection. Seventy percent of PCR-positive cases had died from diseases, which produce secondary immunodeficiency (cancer,

malnutrition, autoimmunity), and thus would have had a high risk of acquiring tuberculosis.

*In situ* PCR has become a valuable tool in the analysis of intracellular DNA and RNA sequences (Embreton, *et al.*, 1993, Nuovo, 1994). In this study, the power and specificity of *in situ* PCR was employed to identify the cells harbouring MTB DNA during latent tuberculosis infection. All necessary precautions were taken to avoid false positives. Any non-specific product DNA, formed by polymerase-associated DNA repair was detected by including a duplicate of each section tested to which no primers were added. False positives due to contamination of PCR reagents were avoided using negative controls to which all the PCR reagents were added. Digoxigenin-11-dUTP was incorporated into the PCR protocol to prevent diffusion of amplicon DNA into neighbouring uninfected cells, and MTB DNA was not detected in the post-amplification PCR mix.

Even though, several *in vitro* studies have shown that MTB can enter and multiply within non-professional phagocytic cells, for the first time using *in situ* PCR this study has identified the presence of MTB DNA in non-professional phagocytic cells *in vivo* in people who died from causes other than TB. Alveolar and interstitial macrophages were the most commonly positive cells, but positivity was seen in type II pneumocytes, endothelial cells, and fibroblasts, particularly in cells located in or close to the subpleural fibrotic bands with variable deposition of carbon particles. Recently, using the same methodology employed in this study, a similar observation was made in an experimental latent tuberculosis model in C57BL/6 x DBA/2 F1 hybrid mice (Arriaga, *et al.*, 2002).

Pathogenic mycobacteria have evolved mechanisms to rapidly invade monocytes and macrophages. Recently, several *in vitro* studies have shown that they are also capable of interacting with a variety of host cells, such as fibroblasts, endothelium, and human epithelial cells (Bermudez, 1991, Bermudez and Young, 1994, Mehta, *et al.*, 1996) (Bermudez and Goodman, 1996) (Mapother and Songer, 1984, Ramakrishnan and Falkow, 1994, Sheridan, *et al.*, 1994). The present study and the observation made by Arriaga and colleagues indicate that similar phenomena also occur *in vivo*. Earlier it was assumed that the MTB bacilli,

after internalisation by alveolar macrophages, were transported into the blood stream from the hilar lymph nodes through the thoracic duct. However, a recent study published by Bermudez and colleagues has shown that MTB but not *M. bovis* BCG cross the alveolar barrier by invading the alveolar epithelial cells and secondarily the endothelial layer. MTB invades this entire layer either within macrophages or on its own (Bermudez, *et al.*, 2002). These investigators also noted that monocytes crossed the barrier with greater efficiency when alveolar epithelial cells were infected with MTB than when they were not infected. This is because infected alveolar epithelial cells trigger the release of chemokines and thereby create a gradient responsible for the migration of infected mononuclear phagocytes. These observations further support the possible localisation of tubercle bacilli in non-professional phagocytic cells in the lung.

The observation of the presence of MTB DNA in non-professional phagocytic cells *in vivo* raises many questions. Firstly, why are the infected cells not killed by mycobacteria? Two hypotheses provide a possible explanation.

(1) As the non-professional phagocytic cells are poor in antigen-presenting capacity, there may be no defence exerted by the host immune system on these infected cells. Therefore, there is no pressure on the MTB residing inside the non-professional phagocytic cells compared to the ones in the macrophages. In addition, it was observed in this study that the cells carrying MTB DNA were located in normal looking lung tissue. Therefore, these cells are well away from the pool of cytokines that are usually abundant at the sites of granulomas (Arriaga, *et al.*, 2002). It has been shown that infected non-professional phagocytic cells are highly sensitive to TNF- $\alpha$  (Filley and Rook, 1991, Filley, *et al.*, 1992) and undergo necrosis. Hence, localisation well away from the active site within the non-professional phagocytic cells could be a mechanism MTB uses to persist in the host.

(2) The growth of MTB could be attenuated inside non-professional phagocytic cells. A recent study on intracellular growth of *Salmonella enterica* serovar *typhimurium* supports this hypothesis. Proliferation of the virulent strain of this organism was attenuated when they were cultured with fibroblast cells (Cano, *et*

*al.*, 2001). On the other hand, massive intracellular proliferation was observed predominantly within CD18-expressing phagocytes but not in epithelial cells. These observations raise the question of whether, during the infection, the invasion of non-professional phagocytic cells by an intracellular organism might be followed by adaptation of the pathogen to a slow-growth or resting state. It is not clear at present whether MTB also has a similar growth attenuation response when they are inside non-professional phagocytic cells. Identification of any proteins that are involved in such a type of response would be a potential target to treat latently infected people.

A second question regarding the localisation of MTB in non-professional phagocytic cells is how they gain entry into these cells. Although significant progress has been made in defining the macrophage receptors participating in MTB uptake, and the signalling events involved in the entry of bacilli into macrophages, little is known about the ligand-receptor interaction between MTB and other cell types and about the signalling events in association with uptake of MTB by non-professional phagocytic cells. Furthermore, the fate of MTB *in vivo* after invasion of non-professional phagocytic cells is currently unknown.

A number of ligand-receptor interactions mediate the entry of MTB into macrophages. MTB utilises receptors such as complementary receptors, mannose receptors, surfactant protein receptors, CD14, scavenger receptors, and Fc $\gamma$  receptors, on the macrophage to gain entry into it (Ernst, 1998). Few adhesins on the surface of mycobacteria have been identified, which facilitate adhesion to non-professional phagocytic cells and internalisation. An adhesin for fibronectin has been characterised in a number of species of mycobacteria (Ratliff, *et al.*, 1993) and has been associated with the ability of *M. bovis* to bind to bladder cells (Ratliff, *et al.*, 1987). Another adhesin molecule shown to be involved with the interaction of epithelial cells is heparin-binding haemagglutinin adhesin (HBHA) (Menozzi, *et al.*, 1996). HBHA-mediated adherence is specific for non-professional phagocytic cells. One recent work showed an interaction of MTB with the plasma membrane steroid cholesterol (Gatfield and Pieters, 2000). When macrophages are depleted of cholesterol, MTB cannot no longer enter the macrophages. Therefore, steroid cholesterol in the plasma membrane of non-

professional phagocytic cells may also serve as a receptor for the entry of bacilli. Another protein shown to be essential for invasion of HeLa epithelial cell lines was Mce1, expressed on the surface of the MTB (Arruda, *et al.*, 1993).

Uptake of MTB by the macrophages is associated with a number of early intracellular signalling events and cytoskeletal rearrangements. A study has shown active participation of the mammalian cell lines, HT-29 and HEp-2 in the process of internalisation of *M. avium* (Bermudez and Young, 1994). Microfilament inhibitors inhibit the uptake of *M. avium* by these cell lines. Furthermore, inhibition of Src family proteins, such as tyrosine kinase and serine/threonine kinases, associated with early signalling events, block the entry of *M. avium* into cultured cell lines (Bermudez and Young, 1994). These proteins are necessary for the uptake of MTB by macrophages and play an important role in the membrane remodelling during phagocytosis (Deretic and Fratti, 1999). These observations disclose that non-professional phagocytic cells also actively participate in phagocytosis. However, the phagosome biogenesis and membrane trafficking in infected non-professional phagocytic cells is poorly understood at present.

It is known that bacterial proliferation within cultured epithelial cells correlates with alterations in the distribution of host lysosomal membrane glycoproteins (LGPs) and the formation of filamentous structures. Pathogen interaction with host vacuolar trafficking machinery has also been proposed as a major strategy promoting intracellular growth in *Salmonella enterica* infection (Garcia-del Portillo, *et al.*, 1993, Garcia-del Portillo and Finlay, 1994, Meresse, *et al.*, 1999). Applying the same approach to MTB should disclose mechanisms behind long-term persistence of MTB in non-professional phagocytic cells and thus enable the identification of the virulence factors regulating this function.

The observation of the presence of MTB DNA in apparently normal tissue is rather intriguing, as many *in vitro* studies have proved that the cause, which drives bacilli to shift into a state of non-replicating persistence, is depletion of oxygen. The sites where MTB DNA is located in the present study and in the latent mouse model (Arriaga, *et al.*, 2002) are clearly not anaerobic. This finding

is further supported by the evidence that metronidazole, a drug which acts on anaerobic bacilli, has no effect in the Cornell model of latent tuberculosis (Dhillon, *et al.*, 1998). Perhaps drug may have got in to the bacilli, but their anaerobic metabolism may not be similar to that of other anaerobic bacteria in which metronidazole is effective.

In conclusion, identification of MTB DNA within non-professional phagocytic cells located well away from the active tuberculous lesions *in vivo* has opened up a new and exciting field of research, which could accelerate the search for strategies for elimination of latent infection.

## Chapter 7

### 7. Organ distribution and cellular morphology of tubercle bacilli during latent tuberculosis infection

#### 7.1 Metastatic localisation and intra-patient strain diversity of *Mycobacterium tuberculosis* complex in people without clinical disease

##### 7.1.1 Introduction

*Mycobacterium tuberculosis* is capable of colonising many sites in the body eliciting diseases ranging from lymphadenitis, skeletal tuberculosis, genitourinary tuberculosis, tuberculous meningitis, and disseminated miliary tuberculosis. This range of so-called “extra-pulmonary” tuberculosis infection accounts for about 15% of the total TB cases (Lam and Lo, 2001). However, the frequency of extra-pulmonary disease is diverse in different populations (Snider, 1975, Kennedy, 1989, Rowinska-Zakrezewska, *et al.*, 1995).

The most probable routes of entry of tubercle bacilli into the human body are respiratory and gastrointestinal. Therefore, involvement of any other organs, except lymph nodes associated with respiratory and gastrointestinal systems, is certainly due to reactivation of tubercle bacilli disseminated during the course of primary infection. MTB DNA was detected in the blood from 39 of 41 of pulmonary tuberculosis patients (Condos, *et al.*, 1996), which provides evidence that mycobacteria can enter the circulation. In most cases of extra-pulmonary disease, at the time of presentation there is no evidence of active pulmonary disease, although there may be clinical or radiological evidence of past infection (Eastwood, *et al.*, 2001). One recent study on transplant patients has revealed transmission of TB to the recipients through the donor organ (Graham, *et al.*, 2001). Evidences from guinea pig animal models also support the dissemination of tubercle bacilli to distal organs during primary infection (Balasubramanian, *et al.*, 1994a). Therefore, there is every reason to believe that latency is maintained in widely disseminated sites rather than being confined to the lung.

Mice infected intravenously or by aerosol have shown dissemination of infection to other organs. However, the proliferation of bacilli varies according to the organ

in which bacilli are lodged. Bacilli in the liver, for example, do not grow as fast as the bacilli in the spleen (Orme and Collins, 1994). A retrospective study of large number of autopsies of Hong Kong people disclosed that 30% of cases of tuberculosis were extra-pulmonary. Hong Kong is a TB-endemic area where the annual incidence is estimated to be 110 per 100, 000. The five most common extra-pulmonary sites of tuberculosis in these autopsies were the liver, spleen, kidney, bone, and adrenal gland (Lam and Lo, 2001). Even though, mouse models showed that tubercle bacilli grow slowly in the liver compared to other organs, the most common organ involved in extra-pulmonary tuberculosis infection among these Hong Kong people was the liver.

The specific sites of latency and persistence of MTB, in terms of both organ and cell type remain elusive despite the extensive investigations into the mechanisms by which tubercle bacilli persist. Conventional PCR and *in situ* PCR have enabled the identification of those cadavers of Mexicans who harboured MTB DNA in their lung (Hernandez-Pando, *et al.*, 2000). In the present study, the same method was employed to study the metastatic location of latent tuberculosis infection in those individuals who were positive for MTB DNA in their lung (described in chapter 6) and, if such sites were found, to pinpoint the cellular location of MTB DNA in different anatomical structures.

If any MTB DNA isolated from different anatomical sites, the second aim of this study was, to investigate the intra-patient strain diversity.

### **7.1.2 Materials and methods**

#### **7.1.2.1 Case selection**

Paraffin-embedded specimens from multiple anatomical sites of six cadavers with lungs positive for MTB DNA, detected by both conventional and *in situ* PCR in the study described in Chapter 6, were selected to examine the organ distribution of MTB DNA. Multiple anatomical sites from three cadavers in which lungs were negative for MTB DNA were also tested. Organs from three cadavers of Mexicans who died because of pulmonary tuberculosis were also analysed to study the involvement of extra-pulmonary sites during the active disease.

### **7.1.2.2 Experimental details**

Sections were stained using haematoxylin and eosin for histological examination. Conventional PCR and *in situ* PCR as described in Chapter 2 were employed with primers from the IS6110 element to detect MTB DNA. DNA extraction using GuSCN/silica suspension was not sufficiently efficient to detect MTB DNA in some anatomical sites, especially in tissues from the kidney. In these cases the Qiagen DNeasy® tissue kit was used to extract DNA from kidney tissues.

Spoligotyping was performed on the DNA extracts from all anatomical sites. Those DNA extracts giving ambiguous spoligotype patterns were analysed further for *M. bovis* DNA using *M. bovis* specific primers designed by Dr. Helen Donoghue (Chapter 2).

### **7.1.3 Results**

#### **7.1.3.1 MTB DNA detection by conventional and *in situ* PCR**

With the exception of one case (case 6), MTB DNA was identified at different anatomical sites of patients whose lungs were positive for MTB DNA (Table 7.1). The most common extra-pulmonary sites positive for MTB DNA were liver, spleen, and kidney. Tissue from the spinal medulla of a 15 year-old male child who died due to acute lymphatic leukaemia was identified as harbouring MTB DNA. *In situ* PCR revealed MTB DNA in tissue from the pons in case 3. In cases 4 and 6 whose deaths were due to causes not related to conditions that affect the immune response, MTB infection was found mainly restricted to lung, kidney and spleen; and lung; respectively.

Both PCR methods revealed the sporadic presence of MTB DNA in extra-pulmonary sites of patients who died from pulmonary TB (Table 7.2). None of the extra-pulmonary sites of pulmonary TB cases showed any histological disease involvement. However, MTB DNA was detected in the cerebellum of case 7 and mesencephalus of case 8, which is a pathogenic indicator. MTB DNA was found in either colon or small intestine of all three pulmonary tuberculosis patients.

The cerebellum, right, and left kidney, spleen, and liver were examined for MTB DNA in those who were negative for MTB in their lung. Except for the spleen from one case, who was a 58 years old female who died of liver cirrhosis, all other anatomical sites were negative in those who were negative for MTB DNA in their lung.

Cases \		1		2		3		4		5		6	
Organs	Method	A	B	A	B	A	B	A	B	A	B	A	B
Lung	+	+	+	+	+	+	+	+	+	+	+	+	+
Cerebral cortex	-	-	-	-	-	-	-	-	-	-	-	-	-
Cerebellum	-	-	-	-	-	-	-	-	-	-	-	-	-
Frontal cortex	-	-	-	-	-	-	-	-	-	-	-	-	-
Basal nucleus	-	-	-	-	-	-	-	-	-	-	-	-	-
Hippocampus	-	-	-	-	-	-	-	-	-	-	-	-	-
Pons	-	-	-	-	-	+	-	-	-	-	-	-	-
Bulbar	-	-	-	-	-	-	-	-	-	-	-	-	-
Spinal medulla	-	-	+	+	-	-	-	-	-	-	-	-	-
Choroid plexus	-	-	-	-	-	-	-	-	-	-	-	-	-
Occipital cortex	-	-	-	-	-	-	-	-	-	-	-	-	-
Right kidney	+	+	-	-	-	+	+	+	+	-	-	-	-
Left kidney	+	+	+	+	+	+	+	NT	NT	+	-	-	-
Spleen	+	-	+	+	+	+	+	+	+	NT	NT	-	-
Liver	+	-	-	-	+	+	+	-	-	-	+	-	-
Small intestine	+	-	-	-	-	-	-	-	-	+	+	-	-
Colon	-	-	-	-	NT	NT	-	-	-	+	+	-	-
Adrenal	NT	NT	-	-	+	+	+	+	+	-	-	-	-

Table 7.1: Cumulative data for the metastatic location of MTB DNA in lung positive cases

Clinical features: Case 1: Female 17, malignant tumour of striated muscle; Case 2: Male 15, acute lymphatic leukaemia; Case 3: Female 15, acute lymphoblastic leukaemia; Case 4: Male 13, hydrocephalus, cerebral paralysis; Case 5: Female 12, acute myeloblastic leukaemia; Case 6: Male 12, congenital heart disease

A: Conventional PCR; B: *in situ* PCR

NT: Not tested

Cases	1	2	3	4	5	6	7	8	9
Brain	-	+	+	-	-	-	+	+	-
Right kidney	+	-	+	+	-	-	-	NT	-
Left kidney	+	+	+	NT	+	-	-	NT	+
Spleen	+	+	+	+	NT	-	+	NT	-
Liver	+	+	+	-	+	-	+	NT	-
Small intestine	+	-	-	-	+	-	-	-	+
Colon	-	-	NT	-	+	-	+	+	-
Adrenal	NT	-	+	+	-	-	-	NT	+
Lung	+	+	+	+	+	+	+	+	+

Table 7.2: Cumulative data showing organ localisation of MTB DNA detected by either conventional PCR or by *in situ* PCR in people died from causes other than TB and people died because of pulmonary tuberculosis

Cases 1-6; People died of causes other than TB and positive for MTB DNA in their lung

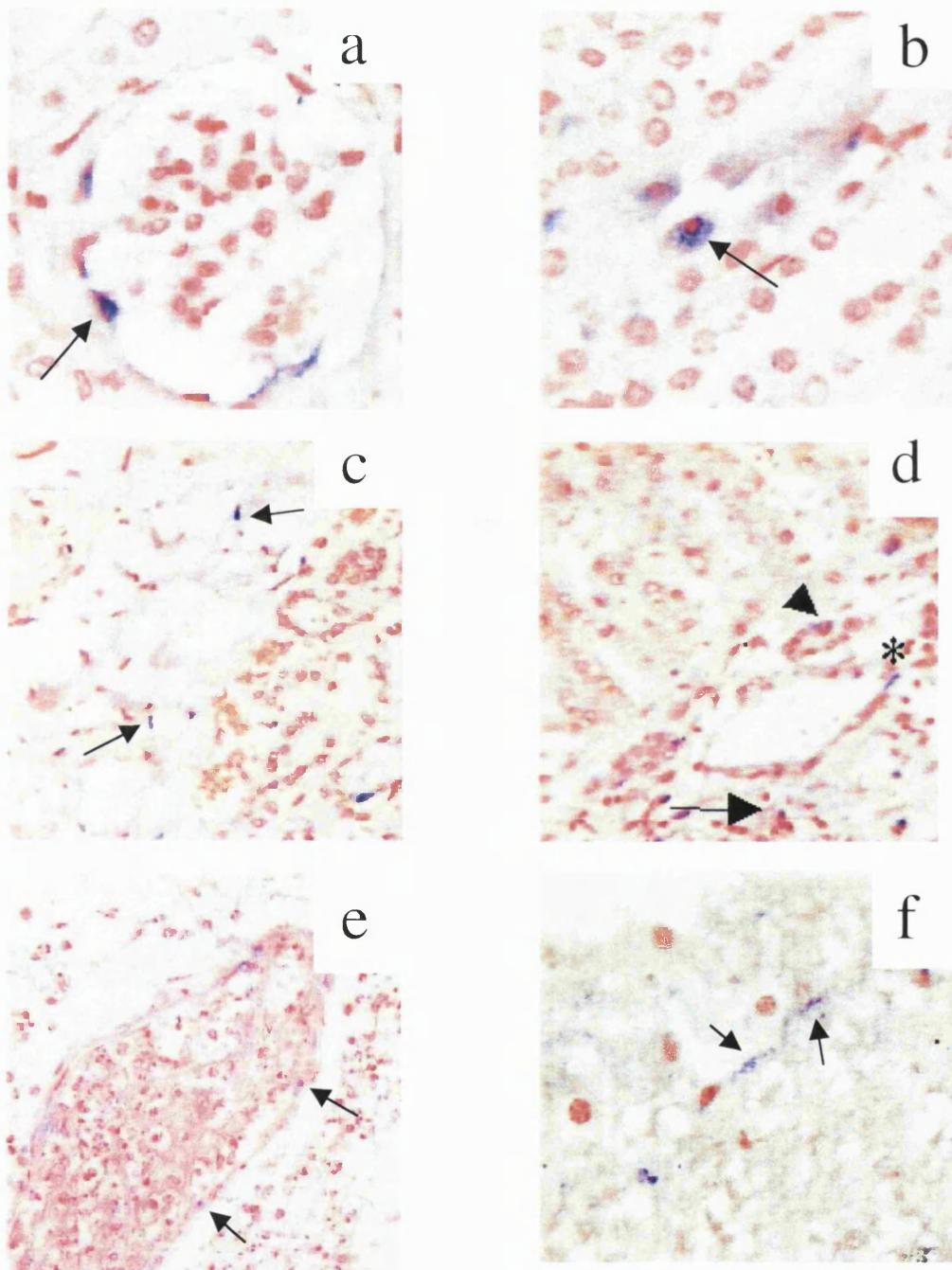
Cases 7-9; People died of pulmonary tuberculosis; NT: Not Tested

Altogether 104 tissue samples from cadavers positive for MTB DNA in their lung, 24 tissue samples from those negative for MTB DNA in their lung and 26 tissue samples from pulmonary TB cases were examined using both conventional and *in situ* PCR. DNA extraction and conventional PCR were performed twice on each block on consecutive sections. MTB DNA was detected in 23% (24/104) of tissue samples from different anatomical sites of those positive for MTB DNA in their lung by one or both methods of PCR. Concordant results by both methods were obtained in 19% (20/104) of samples. Overall, concordance between the conventional and *in situ* PCR results of cadavers positive for MTB DNA in their lungs was 77%. Concordance between the results of both methods obtained in active pulmonary cases was 92% and in those who were negative for MTB DNA in their lungs, it was 96%. Even though MTB DNA was detected in the kidney sections with *in situ* PCR, amplification could not be obtained from kidney sections using the GuSCN/silica suspension extraction method. Nevertheless, DNA was extracted efficiently from kidney sections using Qiagen DNAeasy® extraction kit.

Cellular localisation of positive signals in different anatomical sites is tabulated in table 7.3. The cellular location of positive signals was verified independently by Dr. Rogelio Hernandez Pando, pathologist, Institute of National Nutrition, Mexico City, Mexico. The location and morphology of the positive cells in the kidney sections strongly suggested that these were tubular and Bowman's epithelial cells (Figure 7.1 (a) & (b)). Positive signals were also observed in fibroblasts located in the connective tissue of the kidney pelvic area (Figure 7.1 (c)). Interestingly, in addition to kupffer cells, positive labelling was found in occasional hepatocytes and biliary epithelial cells in the liver (Figure 7.1(d)). In the brain, endothelial cells of blood vessels and occasional endothelial cells of capillaries were positive for MTB DNA (Figure 7.1(e)). The intriguing observation was the positivity in the neuron fibres in the white substance of the brain (Figure 7.1(f)). *In situ* PCR was repeated on the consecutive sections of this block in the laboratory where the thesis author worked and in the Department of Pathology, Institute of Nutrition, Mexico City, Mexico. Consistent results were obtained in both laboratories.

Organ (s)	Cell type(s)
Kidney	Parietal epithelial cells from Bowman's capsule, tubular epithelial cells, fibroblasts embedded in the connective tissues
Liver	Kupffer cells, hepatocytes, biliary epithelial cells, endothelial cells in portal vein, fibroblasts in hepatic portal area
Spleen	Macrophages, endothelial cells from spleen red pulp
Colon/Small intestine	Intestinal epithelial cells, Fibroblasts, macrophages and endothelial cells of capillaries from the intestinal submucosa
Adrenal gland	Fibrous capsule in fibroblasts, epithelial adrenal cells from the cortex, and occasional endothelial cells in cortical sinusoids
Brain	Endothelial cells of large blood vessel, occasional endothelial cells of capillaries, neurons in white matter

**Table 7.3: Cellular localisation of MTB DNA in different anatomical site**

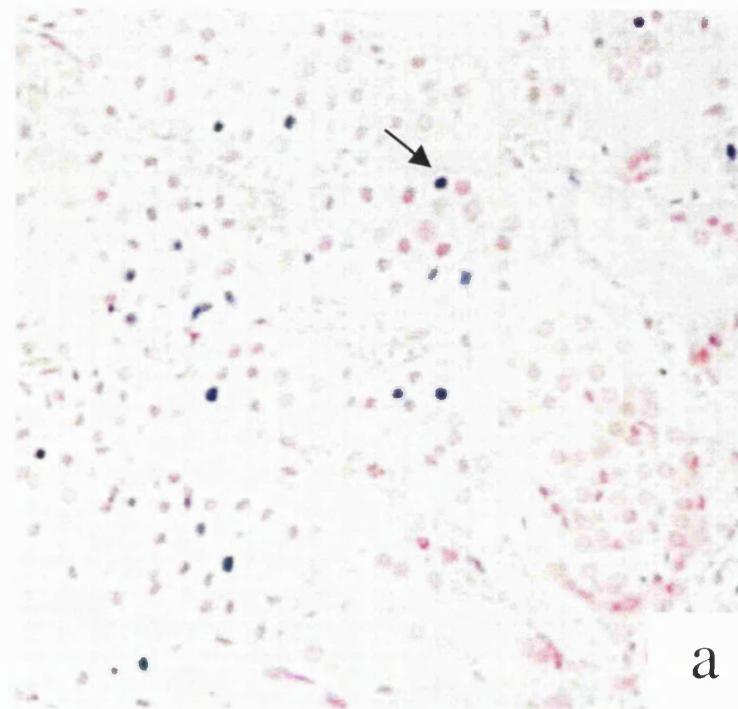


**Figure 7.1: Representative micrographs of the cellular localisation of MTB DNA revealed by *in situ* PCR in different anatomical sites from different cadavers that were positive for MTB DNA in their lung.**

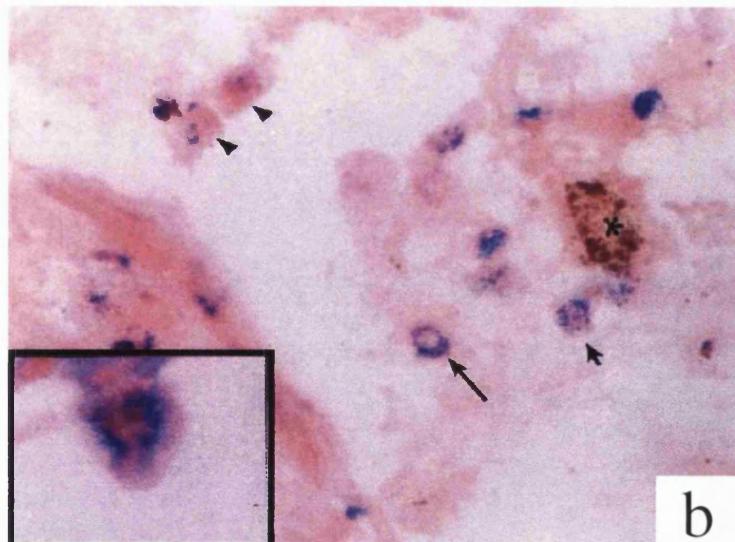
a=Parietal epithelial cells of Bowman's capsule showing positive (purple) labelling (black arrow). b=Tubular cells from kidney medulla showing labelling (black arrow). c=Positive fibroblasts embedded in the connective tissue from the pelocaliceal area of the kidney (black arrow). d= Some hepatic portal areas have positive fibroblasts (arrow), biliary epithelial cells (arrow head), and endothelial cells from portal vein (asterisk) showing positive labelling. e=Endothelial cells from a large blood vessel in the basal brain showing mycobacterial DNA positivity (black arrow). f=Few neuron fibres from the brain white substance showing mycobacterial labelling (black arrow)

Original magnification X 400

Another fascinating observation of this study was the detection of occasional non-professional phagocytic cells from different anatomical sites of cadavers whose lung were positive for MTB DNA showing clear labelling inside the nucleus (Figure 7.2). In most of the positive cells MTB DNA was detected in the cytoplasm, however in a few cells from different anatomical sites MTB DNA was detected inside the nucleus.



a



b

Figure 7.2: Representative micrograph of the medulla region of adrenal showing nuclear localisation of MTB DNA (arrow) (a). and positive labelling in the cytoplasm of macrophages in the lung (b).

### 7.1.3.2 Spoligotype pattern of DNA extracts

DNA extracts of different anatomical sites which were positive for MTB DNA using MTB-specific primers from the IS6110 element either by conventional or by *in situ* PCR were spoligotyped. Table 7.4 showing the spoligotype pattern of MTB DNA extracted from people who died from pulmonary tuberculosis. None of the DNA extracts from different anatomical sites from the same patient revealed an identical pattern. Comparison of the spoligotype pattern of the three pulmonary tuberculosis cases presently studied with the international database of spoligotype patterns at the National Institute of Public Health and Environment (RIVM), The Netherlands revealed patterns similar to previously described MTB isolates.

DNA source	Spoligotype pattern
H37Rv	777777747760771
P3	67677377777600
<b>Case 7</b>	
Lung	361277477740200
Colon	317340006660611
Liver	777777777760771
Spleen	660000007760671
Cerebellum	620343021760661
<b>Case 8</b>	
Lung	000000006360571
Colon	77777775760771
Mesencephalus	Incomplete
<b>Case 9</b>	
Lung	777776205760771
Kidney	77777777521717
Small intestine	057777270000000

**Table 7.4: Spoligotype pattern of DNA extracted from different necropsy sites obtained from people who died from pulmonary tuberculosis.**

In contrast to this, very unusual spoligotype patterns were observed when the DNA extracts obtained from people who died from causes other than TB were

typed (Figure 7.3). Three DNA extracts (lanes 4, 12, and 18) show a truncated pattern. Almost all other samples reacted with all the 43 spacers. However, the intensity of the spots varied in each pattern. Comparison of these patterns with the international database revealed similar patterns (reaction with all 43 spacers) in two *M. bovis* isolates, both isolates from South Africa. The source of one of these isolate was a lion. *Mycobacterium tuberculosis* isolated from an elderly women born in 1927 also showed a similar pattern. One isolate from a TB patient from Hawaii showed a pattern similar to those observed in the present study, which lacked spacer 33. A Spoligotype pattern with a deletion in spacer 34 has not been reported previously. As a spoligotype pattern similar to *M. bovis* was found, those giving this similar pattern were analysed for *M. bovis* DNA using specific primers (Chapter 2). None of the DNA extracts was positive for *M. bovis*.

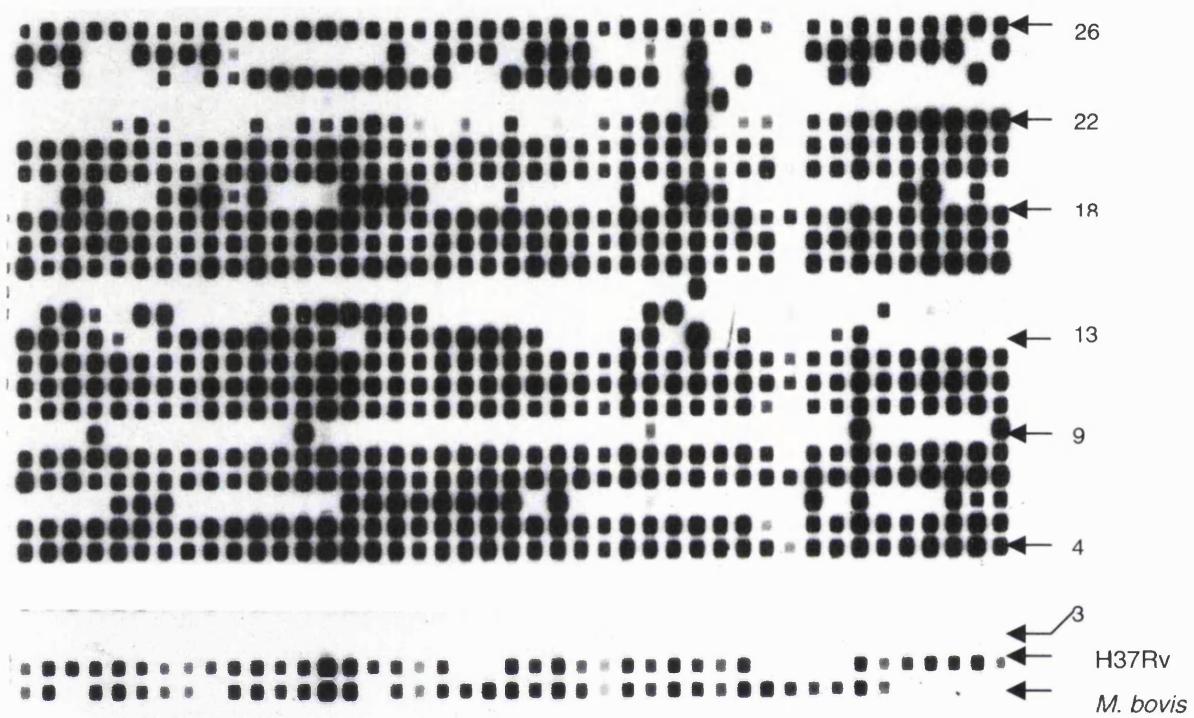


Figure 7.3: Spoligotype patterns of *M. tuberculosis* complex using DNA extracted from different anatomical sites of people who died from causes other than TB. Lanes: 1, *M. bovis* BCG; 2, H37Rv; 3, negative control; 4 to 9, right kidney, left kidney, spleen, liver, small intestine, and lung of case 1; 10 to 13, spinal medulla, spleen, kidney and lung of case 2, 14-18, spleen, kidney, liver, adrenal gland, and lung of case 3; 19-22, spleen, kidney, adrenal gland and lung of case 5, 23-26, small intestine, colon, kidney and lung of case 6.

#### 7.1.4 Discussion

It is widely accepted that tuberculosis of extra-pulmonary sites, except gastrointestinal and lymph nodes associated with the gastrointestinal and respiratory system, is due to reactivation of latent tuberculosis infection. To my knowledge, no formal study has attempted to show that latency is maintained at different anatomical sites of latently infected people. For the first time, using conventional and *in situ* PCRs, this study has examined tissues from different anatomical sites of people who died from causes other than TB, but who were positive for MTB DNA in their lung, to detect specific sites and cellular location of MTB DNA, possibly tubercle bacilli, during latent tuberculosis infection. Nevertheless, a few previous studies have examined clinical specimens representing different anatomical sites of pulmonary tuberculosis patients (Mortier, *et al.*, 1996, Actei, *et al.*, 1999) (Bentz, *et al.*, 1975, Lourenco, *et al.*, 2000). Aceti *et al.* (1999) demonstrated the presence of MTB DNA in the urine of all 13 HIV-infected active pulmonary cases included in their study. Mortier *et al.* (1996) and Bentz *et al.* (1975) isolated MTB in culture of urine samples obtained from 5-8% and 4.7% of active pulmonary HIV-negative patients, respectively (Bentz, *et al.*, 1975, Mortier, *et al.*, 1996). MTB has been detected in the blood from 39 of 41 patients with proven TB using DNA amplification (Condos, *et al.*, 1996). These findings provide evidence that during active pulmonary tuberculosis mycobacteria can enter the blood circulation and disseminate to distant organs. However, in the present study, strains different from the one present in the lung were detected in different anatomical site of the same patient. This observed discrepancy is discussed later. In the present study of four samples of kidney tissues from two pulmonary TB cases analysed, one was positive for MTB DNA. It is therefore imaginable that tubercle bacilli are cleared from the blood stream in the urine. The present study revealed that the same phenomenon could occur during the primary infection as well, because MTB DNA has been detected in distant organs of people who were positive for MTB DNA in their lung, but not in those who were negative for MTB DNA in their lung. Of 11 tissue samples from the kidneys of six cadavers identified as positive for MTB DNA in their lung, seven were positive for MTB DNA. Bacterial clearance from the blood stream in the kidney glomerulus is evident, as MTB DNA was located in the epithelial cells from Bowman's capsule and renal tubules.

MTB-positive cells were also located in the liver, spleen, adrenal gland, gut, and brain. MTB DNA was located in the different anatomical sites in tissue-specific macrophages, in epithelial and/or endothelial cells of blood vessels, fibroblasts and epithelial cells of the kidney glomerulus and biliary duct. MTB-positivity of tissue-specific macrophages suggests that migration of infected monocytes from the primary infection sites and subsequent differentiation into tissue-specific macrophages may serve to disseminate latently infected cells into the periphery during the primary infection.

Shepard first showed that MTB could enter not only HeLa cells but also human amnion and monkey kidney (Shepard, 1956). *In vitro* studies using endothelial cells from spleen, kidney, lung, and bone marrow have demonstrated that endothelial cells in these areas may phagocytose microorganisms and ingest polystyrene beads (Ryan, 1984). It is not surprising to detect MTB DNA in fibroblasts of connective tissues, as adhesin for fibronectin has been characterised in a number of species of mycobacteria (Ratliff, *et al.*, 1993). Furthermore, for the past twenty years *M. bovis* BCG immunotherapy has been used to treat superficial bladder cancer. This is based on the principle that it binds to fibronectin on fibroblasts, is internalised, and inhibits tumour growth (Zhao, *et al.*, 2000).

As mentioned in chapter 6, neither the mechanism of entry of MTB into non-professional phagocytic cells nor the fate of bacilli after entry is understood at present. Entry of MTB into non-professional phagocytic cells such as endothelial cells may also facilitate dissemination. It has been shown that endothelial cells acutely infected with cytomegalovirus, a member of the herpes virus family, enlarged and detached from the vessel wall and entered the blood stream, causing viral dissemination (Grefte, *et al.*, 1993, Percivalle, *et al.*, 1993). It is not known whether this is the case with MTB-infected endothelial cells. If it is the case, and these infected endothelial cells escape the toxic effect of cytokines, this may partially explain the demonstration of MTB DNA in the capillaries of brain.

The presence of MTB DNA in neurone fibres is a really intriguing observation. A closely related species to MTB, *M. leprae*, is well known for its capacity to enter the axons of the peripheral nervous system and cause nerve damage (Rambukkana, 2000). However, it has not been known to affect the central nervous system. Therefore, the detection of MTB DNA in the neurone fibres warrants further investigation as neurological complications have been noted in some tuberculous meningitis patients. The observation of neurone fibre-positivity for MTB DNA in the present study makes it tempting to hypothesise, that neurological complications that occur in a fraction of tuberculous meningitis patients may be due to persistence of MTB in the neurones in the central nervous system.

Another fascinating observation of this study was the presence of positive labelling inside the nucleus of human cells. At present it is not clear whether the MTB DNA found inside the nucleus is integrated into the human genome or found in an episomal form. Many viruses enter a latent state, in which their genomes are copied many times into host cells and stored so that they cannot be detected at any time by standard virologic methods (Alberts, *et al.*, 1994). The observation of positive labelling in the nucleus tempts one to suggest that tubercle bacilli may also adopt such a mechanism during latent infection.

In the present study, 80% and 66% of spleen and liver tissues, respectively, from people latently infected in their lung were positive for MTB DNA. A study on large numbers of autopsies of people who died from tuberculosis in a TB endemic area has revealed that the highest incidence of extra-pulmonary tuberculosis was in the liver followed by spleen and kidney (Lam and Lo, 2001). Of interest, 70% of cases of extra-pulmonary TB were diagnosed in this group of patients only at autopsy. In the present study, MTB DNA was detected in the gastrointestinal system of all three pulmonary TB cases, indicating bacilli reach these sites with the swallowed sputa and gain entry into the non-professional phagocytic cells at the site.

The direct repeat locus of MTB is a suitable model to study the intra-patient strain diversity and to study the evolutionary genetics of MTB complex DNA isolated from clinical materials. In the present study, when the DNA extracts from different

anatomical sites of people who died of pulmonary TB and those who died of causes other than TB, but who were positive for MTB DNA in their lung were genotyped, two entirely different sets of spoligotype patterns were obtained.

The interesting observation of spoligotype pattern of DNA extracts from latently infected people is the presence of all 43 spacers in almost all the samples. An identical pattern to this has been found rarely among the isolates from active TB cases. This indicates that the bacilli causing chronic infection are certainly genotypically different from those causing the acute infection. Currently, the most widely used genetic marker to differentiate *M. tuberculosis* isolates is the IS6110 insertion sequence. Species diversity of the MTB complex is largely caused by changes brought about by insertion sequences, such as gene knockouts and deletions. This means that the movement of mobile elements is a fundamental process generating genomic variation in this pathogen. The DR locus, another marker used in genotyping of MTB has a hot spot for IS6110 insertion. The intact DR locus (with almost all the spacers present) in the DNA extracts of latently infected people suggests that there is no loss of spacers either due to IS6110 element movement or due to homologous recombination. This observation leads to the tempting suggestion that the bacillus causing chronic infection may be an ancestral strain especially because the loss of spacers is unidirectional (Behr, 2002).

This observation raises the question whether excision or integration of an insertion element from the DR locus contributes to the evolution of bacilli to a virulent form under certain circumstances. Data have been presented showing that a single nucleotide change, that may result in amino acid alteration in proteins or modification of regulatory sequences and a subsequent decrease or increase in expression of protein products, can significantly alter the virulence of MTB to guinea pigs (Collins, et al., 1995)(Kawaoka, 1988). Simonet et al. reported that the invasin gene (*inv*) of *Yersinia pestis* (the cause of plague) is inactivated by a 708bp IS200-like element (Simonet, et al., 1996). Moreover, the site of IS1301 insertion in *Neisseria meningitidis* changes the expression of cell surface sialic acid, a crucial virulence factor in blood and brain infections (Hammerschmidt, et al., 1996). It has been speculated that the stability of the IS6110 element can be influenced by the duration of infection, the growth rate of

the bacteria under different conditions, the size of the bacterial population, and the possibility of multiple infection (de Boer, *et al.*, 1999). Furthermore, a similar sequence to the DR locus has been found in *Haloferax* spp, which has a role in replicon partitioning (Mojica, *et al.*, 1995). If the DR locus in MTB has the same function, any changes in the reading frame would affect bacterial replication. Thus, it is reasonable to anticipate that systematic control of excision or integration of mobile elements by MTB can alter the host-pathogen interaction.

However, an alternative explanation for reaction with oligonucleotides of all DR spacer regions in the DNA extracts from latently infected people might be due to multiple strain infection. It is always possible for these people to be exposed to more than one strain, especially in a high incidence community. The speculative scenario of multiple infections raises interesting hypotheses:

- (1) that multiple strains can lurk in the body and during active disease only one strain that cannot co-exist with the host may multiply and cause the infection. The isolation of a single strain from the majority of TB patients would support this hypothesis (de Boer, *et al.*, 1999). This implies that there is an association between the virulence of the strain and genetic susceptibility of the host. Nevertheless, there is no evidence available to support the hypothesis that susceptibility to different strains varies in an individual. However, the involvement of various human genetic factors has been identified in determining susceptibility to tuberculosis (Davies and Grange, 2001).
- (2) that reactivation of one strain can alter the immune balance, which can subsequently lead to reactivation of the other latent 'original' strains.
- (3) that reactivation of an endogenous strain may lead to diminished local immunity, which can allow an exogenous strain to establish infection.

The second and the third hypotheses could account for the observed multiple strain infection in some patients (du Plessis, *et al.*, 2001, Richardson, *et al.*, 2002), and for the isolation of different strains in some of the relapse cases. Restriction fragment length polymorphism (RFLP) of strains isolated from some patients has revealed variance in the band intensities, suggesting simultaneous infection with more than one strain (Pavlic, *et al.*, 1999, Yeh, *et al.*, 1999). Mixed-

strain infections have been described in TB high-prevalence settings such as Madrid, the Spanish prison system and South African mines, often corresponding to human immunodeficiency virus positivity (Chaves, *et al.*, 1999, Godfrey-Faussett, *et al.*, 2000). However, deciding whether a multiple strain infection is due to endogenous reactivation or due to exogenous re-infection with a new strain is difficult.

Comparison of the spoligotype patterns of MTB from latently infected people with the international database at RIVM identified a few identical patterns in *M. tuberculosis* and *M. bovis*. To clarify this further, DNA extracts were analysed for *M. bovis* DNA using *M. bovis*-specific primers (Chapter 2). None of the DNA extracts was positive. However, in Mexico a majority of renal tuberculosis (about 60%) is caused by *M. bovis* (Hernandez Pando, R. personal communication). The primers employed to identify *M. bovis* DNA were designed to span the deletion in region RD7. Perhaps the *M. bovis* strains in Mexico have not undergone deletion at this region. There are a few other genomic tools available, which can differentiate MTB from *M. bovis*. A variation in the pyrazinamidase gene (*pncA*) and *oxyR*, oxidative response regulator gene distinguishes bovine from human isolates (Sreevatsan, *et al.*, 1996, Spainso de los Monteros, *et al.*, 1998). A single base pair variation in *pncA* gene: a guanine residue at this position is characteristic of MTB, rather than the adenine in *M. bovis* (Sreevatsan, *et al.*, 1996). A cytosine residue in the *oxyR* gene of MTB, rather than the Guanine residue in *M. bovis* has been used in many studies to differentiate *M. bovis* from MTB (Taylor, *et al.*, 1999, Sales, *et al.*, 2001).

In contrast to the spoligotype pattern of bacilli in latently infected people spoligotype patterns of DNA extracts from different anatomical sites of TB patients resembled those previously described from TB patients. Although it would be anticipated that identical DNA isolates would be obtained from different anatomical sites in a single individual, none of the spoligotype patterns from the different anatomical sites in the same patient were found to be so in the present study. It is not clear whether the difference apparent in the spoligotype profile represents a genuine difference or an incomplete amplification of the whole DR

region in these samples due to inhibitors in the DNA extract, to a low concentration of DNA or to fragmented DNA.

If this is a genuine result, then the observed diversity in the spoligotype profile may reflect either multiple strain infection or a gradual change of bacterial population at different anatomical sites over time due to e.g. transposition of *IS6110* (de Boer and van Soolingen, 2001).

Another recent study has compared isolates obtained from more than one pulmonary lesion and different anatomical locations of post-mortem samples, using DNA fingerprinting of HIV-negative people who died because of disseminated and pulmonary tuberculosis (du Plessis, *et al.*, 2001). Out of 13 cases studied, two patients were infected with two distinct strains. It should be noted that in the above-mentioned study, MTB isolates not the DNA extracts of the tissue from different sites were spoligotyped. Any bacilli that did not grow on culture, which is likely for bacilli in the latent phase of infection, would have been overlooked.

The major limitation of spoligotyping and RFLP genotyping is their inability to distinguish patterns due to single strain infection from multiple strain infections. Patterns of low-intensity bands were observed in *IS6110* RFLP of DNA extracts obtained from a single colony, indicating mixed strains (de Boer, *et al.*, 2000). However, if different strains are present in an equal ratio, intensity of the bands in RFLP and spots in spoligotyping cannot be used as an indication to identify multiple strain infection.

Specific growth conditions play a role in the occurrence of *IS6110* transposition (de Boer, *et al.*, 1999), which can cause changes in the spoligotype pattern (Groenen, *et al.*, 1993). Analysis of repeated isolates obtained over time from a large population of pulmonary and extra-pulmonary patients has revealed that among patients with extra-pulmonary disease RFLP pattern that differed from the initial isolate were observed frequently. This suggests that disseminated bacilli from the initial infection site gradually change to adapt themselves to various growth conditions. This again implies the role of the *IS6110* and similar insertion elements in the modification of the MTB genotype during adaptation.

Finally, the data obtained through this study re-confirm that tubercle bacilli can lurk at extra-pulmonary sites, not only in tissue-specific macrophages but also in non-professional phagocytic cells. Brain is an immune privileged site where immune responses fail to occur as the anatomical blood-tissue barrier (Blood brain barrier) blocks entry of lymphocytes into the brain tissue. Identification of MTB DNA in the brain tissue of latently infected people warrants an explanation for the dissemination of tubercle bacilli to brain. If MTB-infected endothelial cells can circulate as cytomegalovirus does, this may be a possible route for the bacilli to access brain. A conserved spoligotype pattern of bacilli from latent phase infection is a fascinating observation. It is apparent from the present study that the transposition of IS6110 may play an important role in modifying gene expression during adaptation of bacilli to the latent phase of infection. Exploration of the sites of chromosomal integration or excision of IS6110 may disclose possible changes in the virulence of MTB, and subsequently may reveal the mechanisms behind persistence.

## **7.2 Preliminary study on development of methods to visualise cellular morphology of persisting tubercle bacilli during latent tuberculosis infection**

### **7.2.1 Introduction**

Cellular morphology of *M. tuberculosis* has been a subject of debate since Hans Much first published his rather controversial observation 95 years ago. The organisms described by Much in tuberculous tissues were then referred to as Much's granules (Minchin, 1927, Stanford, 1987). He found these granules especially in tuberculous abscesses where it is difficult to find classical bacilli. They were not properly acid-fast and were not easy to grow on the media normally used for culturing mycobacteria. Interestingly, when they were injected into animals, they failed to give rise to typical tuberculosis disease, but when the tissues were taken from the first animals and injected into a second, that animal developed tuberculous lesions containing classical bacilli.

Existence of Much's granules fell into disrepute because of the strange claim later made by Much that they were responsible for the destruction of onion leaves and stunt in tomato plants (Minchin, 1927). However, detection of ultra-fine forms in membrane-filtered homogenates prepared from the open cavities in the lungs of treated TB patients by electron microscopy observed, by Russian scientists, brought attention back to the existence of Much's granules (Khomenko, 1987). These investigators noted a pronounced morphological variation of the mycobacterial population during anti-tuberculosis treatment. Furthermore, others have observed sputum negativity on microscopy and culture examination even before the closure of the cavity in tuberculosis patients (Fox, 1981). In the latent animal models, tissues from 'sterile state' mice were also negative for MTB on microscopy and culture (McCune, et al., 1966a, McCune, et al., 1966b), but reactivation of disease occurred later. These observations raised the question whether the bacilli, which persist after treatment and natural infection exists in a different morphological form that has lost its acid-fast character because of biological changes in the properties of the cell wall.

In this project, *in situ* PCR on tissue sections from people who died from causes other than TB enabled the identification of tissues, which were positive for MTB DNA, possibly persisting tubercle bacilli. This provided an opportunity to study the cellular morphology of the bacilli that persist during latent tuberculosis infection. Therefore, in this study, an adjacent section to the section that was positive for MTB DNA was examined for tubercle bacilli using acid-fast, auramine and immunohistochemical staining, with the view that if any cells with tubercle bacilli were identified these could be processed for electron microscopy using a Laser-MicroBeam System (PALM®). This system enables the micro-dissection of single cells. In the present study, this technique was also employed to isolate cells identified positive for MTB DNA using *in situ* PCR in order to visualise the morphology of the tubercle bacilli, if this is possible after the processing of tissue for *in situ* PCR.

## 7.2.2 Materials and methods

### Staining of tissue sections

Adjacent sections to the sections that were positive for MTB DNA by *in situ* PCR were stained in order to visualise any bacilli. Sections of a lung from a pulmonary TB case were used as a positive control for all the staining methods. All the staining procedures are described in detail in chapter 2.

### Micro dissection and isolation of single cells using Laser-MicroBeam System (PALM®)

Laser-MicroBeam System (PALM®) enables the micro-dissection of single cells with the aid of a laser beam. A single precisely aimed laser shot ejects the laser-isolated samples from the slide and catapults them directly into the cap of a common microfuge tube (Figure 7.4). This results in empty patches in the tissue section, exactly corresponding to the micro-dissected templates. This laser pressure catapulting technology was employed in the present study with the intention that isolation of single cells harbouring MTB DNA could be further processed for electron microscope to study the cellular morphology. Glass-slide-mounted and membrane-mounted slide-mounted specimens were used for *in situ* PCR. Membrane-mounted slides are the ones where a 1.35 µm thin, stretched polyethylene foil, (P.A.L.M Microlaser Technologies AG) is attached to an objective slide and sections are mounted on the membrane.

This facility is based at P.A.L.M Microlaser Technologies AG, Am Neuland 9+12, 82347 Bernried, Germany. Micro-dissection of *in situ* PCR labelled cells for MTB DNA was carried out by the thesis author at this location for further processing for electron microscopy to study the ultra structure of persisting bacilli.

## PALM Laser-MicroBeam Systems

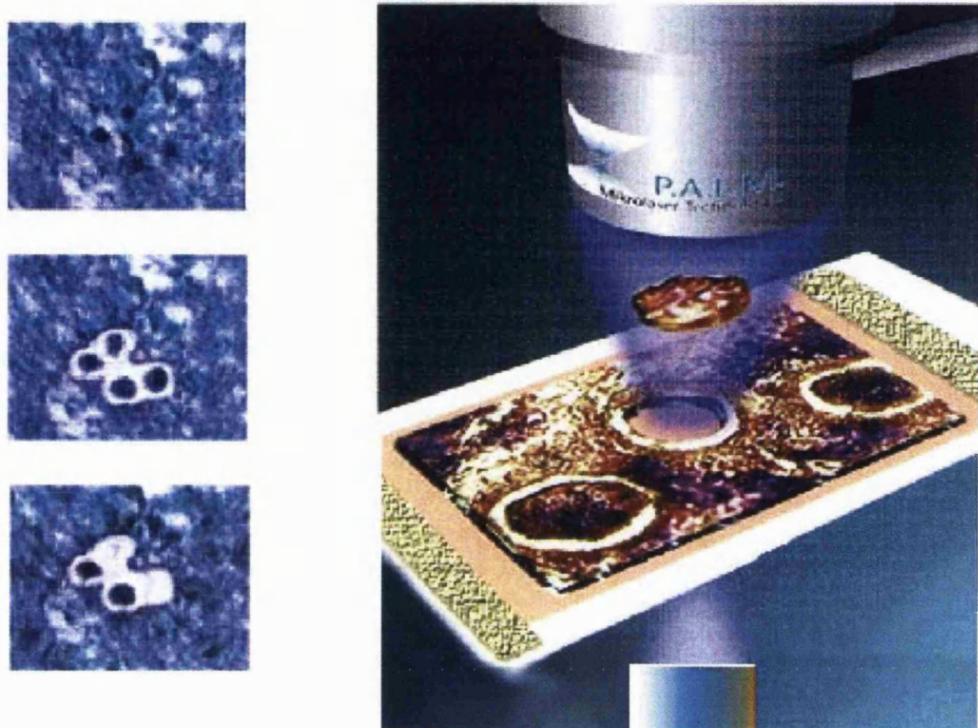


Figure 7.4: Laser-MicroBeam System (PALM<sup>®</sup>)

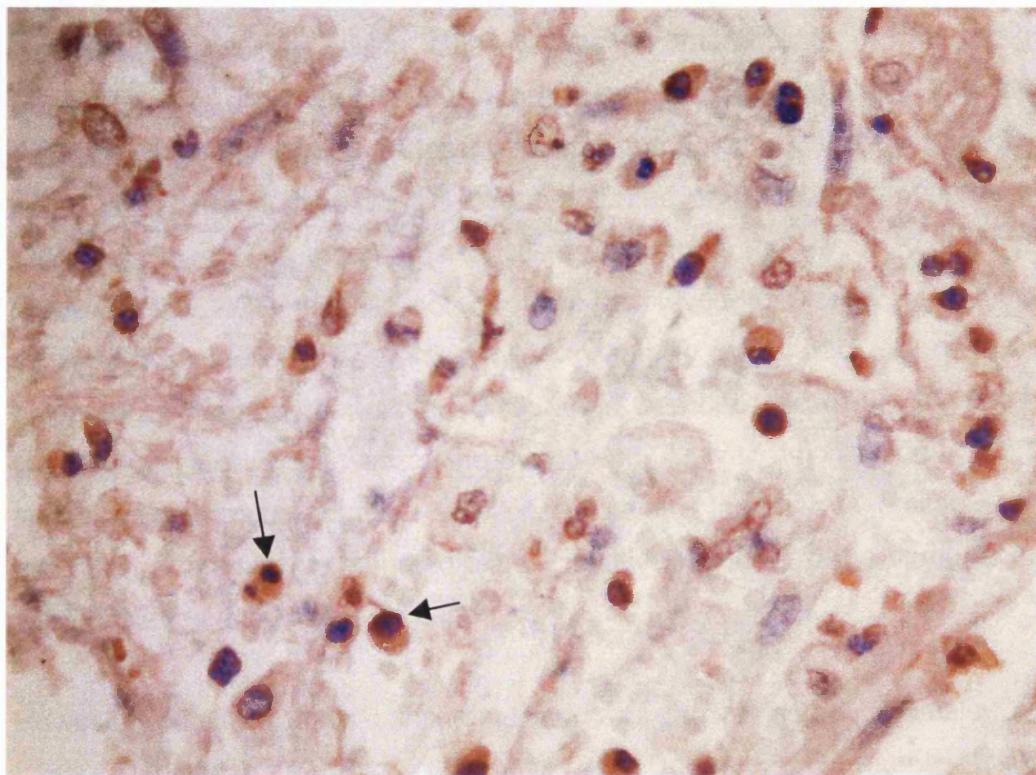
The desired isolated cell is cut out by a high-energy laser beam and ejected from the slide with single precisely pointed laser shots. Owing to the development of extremely high photon density under the specimen of interest (in this case the single cell), the sample is travelling along the light wave front and therefore catapulted directly into the cap of a common PCR reaction tube containing 50 µl of buffer solution. Tube was topped with the cap. After centrifugation, the sample assembles at the bottom of the tube and can be further processed.

### ***In situ* PCR on sections mounted on membrane-mounted slides**

Membrane-mounted slides (P.A.L.M Microlaser Technologies AG) were pre-treated prior to mounting the sections on them. They were first irradiated with 254nm UV in a Bio-Link® UV exposure chamber for 30 minutes to make the membrane hydrophilic. Subsequently slides were coated by dipping them into Poly-L-lysine (Sigma) to fix the membrane firmly to the slide. Slides were then dried at room temperature. Five-micron sections were mounted on these slides and dried overnight at 37°C. Sections were processed for *in situ* PCR as described in chapter 2. A slightly modified PCR amplification programme was used to amplify the target DNA in the sections mounted on the membrane-mounted slides. The thermocycling profile included a single cycle of 95°C for 5 min, 70°C for 2 min, and 72°C for 2 min followed by 37 cycles of 95°C for 1 min, 70°C for 1.5 min, and 72°C for 1 min with an increment of 1 sec/cycle for the denaturing, annealing and extension steps, respectively. The experiment was repeated to check the consistency of localisation of positivity on the adjacent sections.

#### **7.2.3 Results**

Macrophages positive for MTB and mycobacterial antigen were observed in the positive control sections using Ziehl-Neelson and immunohistochemical staining (Figure 7.5) respectively. Auramine staining revealed fluorescing MTB in the positive controls. In contrast, no cells positive for MTB or mycobacterial antigens were seen in sections obtained from tissues of different anatomical sites of people who had died from causes other than tuberculosis. Auramine-stained sections of these tissues were also negative for MTB.



**Figure 7.5: Representative immunohistochemical staining of tissue section of a lung from a pulmonary tuberculosis patient.**

Macrophages in the granuloma showing positive (brown) mycobacterial antigen labelling (arrow).

Initially, *in situ* PCR was performed on tissue sections mounted on glass slides using the PCR amplification protocol described in chapter 2, section 2.8. Micro-dissection and catapultation of positively-labelled cells in the tissue sections resulted in fragmentation. In other words, the morphology of the positively labelled single cells could not be preserved in order to study the cellular morphology of the bacilli. To preserve the morphology of the micro-dissected single cells, a technique using sections mounted on membrane-mounted glass slides was used. The membrane serves as a support and avoids fragmentation. However, the PCR amplification protocol employed to amplify MTB DNA in sections mounted on glass slides failed to amplify target DNA on the sections mounted on membrane-mounted slides. This was because of the alterations in the heat conductivity caused by the membrane. A slight modification in the PCR amplification protocol enabled amplification of the target DNA on the sections mounted on membrane-mounted slides. Positive labelling at the same locations were observed in adjacent sections.

However, due to various constraints, such as the restricted availability of the Laser-MicroBeam System (PALM®) at Bernried, Germany, travelling arrangements and the limited time, further progress could not be made prior to submission of this thesis.

#### 7.2.4 Discussion

For the first time, cellular location of MTB DNA, possibly tubercle bacilli, has been identified in people without clinical disease using *in situ* PCR in this project. This provided the opportunity to study the cellular morphology of tubercle bacilli that persist during latent tuberculosis infection, after the natural infection.

The appearance of a variation in morphology of mycobacteria during anti-tuberculosis treatment is well documented (Khomenko, 1987). Many earlier workers, e.g. Much, Chandrasekhar, Matmann and others have reported existence or isolation of non-acid-fast (NAF) granular forms from tuberculosis lesions or from old cultures (Minchin, 1927, Chandrasekhar and Ratnam, 1992, Mattmann, 2001b). The Cornell mouse model has also provided evidence for the existence of NAF forms in the 'sterile state' mice, where acid fast staining failed

to detect any bacilli, but subsequently cultivatable MTB cells appeared either spontaneously or in response to immune suppression (McCune, *et al.*, 1966a, McCune, *et al.*, 1966b, Flynn and Chan, 2001). The present study also accords well with these observations. Although *in situ* PCR detected MTB DNA, the two staining techniques, Ziehl-Neelsen and auramine, which are widely used to detect mycobacteria in clinical samples, failed to reveal MTB in the sections adjacent to the section that was positive for MTB DNA.

Both staining methods rely on the ability of the bacilli to resist destaining when treated with acid alcohol. The mechanism of acid-fastness is still unknown, although it is most frequently thought to be associated with the lipids and mycolic acid esters present in the cell wall (Draper, 1982). Therefore, failure to detect tubercle bacilli in sections adjacent to the ones that were positive for MTB DNA suggests, that the bacilli persisting during latent tuberculosis infection must be either non-acid-fast or in a cell wall deficient form.

Recently it has been shown that expression of isocitrate lyase, a glyoxylate shunt enzyme, is essential for the bacilli to persist (McKinney, *et al.*, 2000). It was proposed that persisting bacilli, which are thought to be hiding in the granulomas, consume lipids from host-cell debris through the glyoxylate pathway to generate both energy and precursors for carbohydrate synthesis. However, studies described in this thesis have shown that bacilli can persist in areas with normal histology. Therefore, because of the non-acid-fast character of the bacilli in these locations, it is tempting to hypothesis that instead of depending on the host for their nutrients that the persisting bacilli sustain themselves on their own cell wall lipids.

Another possible explanation for the non-acid-fast nature of persisting bacilli may be due to cell wall thickening that was observed in the late log phase of the *in vitro* cultures. Cunningham and Spreadbury have noted the expression of alpha-crystallin protein and its association with cell wall thickening in MTB in the late log phase (Cunningham and Spreadbury, 1998).

Therefore, if the acid-fast nature of the bacilli is due to lipids in the cell wall, disappearance of lipids on the cell wall or/and a greater proportion of proteins in the thickened cell wall may have made them lose their acid-fast nature.

Detection of acid-fast bacilli in the sections adjacent to the sections that were positive for MTB DNA may be unsuccessful due to the property of tubercle bacilli being chromophobic. Prior oxidation with periodic acid followed by application of dye, either magenta or Night blue enabled demonstration of acid-fast bacilli if they were fixed routinely and stained with carbolfuchsin (Nyka, 1963). This staining, too, is acid-fast, but probably involves a different mechanism from the conventional acid-fast stain. In the present study, no attempt has been made to use this staining method to visualise tubercle bacilli in the latently infected tissue because of the limited time.

Failure to detect acid-fast bacilli in the sections adjacent to the sections that were positive for MTB DNA may have resulted because of the atypical nature of the persisting bacilli. Studies have revealed existence of pleomorphic bacilli or cell wall deficient (CWD) forms or L-forms in clinical samples (Mattmann, 2001b).

Cell wall components (LAM and mycolic acid) are the dominant targets of the antibody response to mycobacteria. These are frequent targets for T cells as well. In addition, cell wall components play an important role in triggering expression of cytokines by infected cells (Thole, *et al.*, 1999). Therefore, persisting in a cell wall deficient form may be another mechanism bacilli use, in addition to being away from the infection site (chapter 6), to escape host immune defence. On the other hand, CWD forms may express antigens entirely different from those expressed by typical bacilli MTB. Typhoid carriers were found to carry significant levels of antibody to spheroplasts of *salmonella typhi*, but undetectable antibody levels to the classical form of this organism (Mattmann, 2001a). Thus, evaluation of antibody levels to CWD forms of MTB in latently infected people may be an effective diagnostic method.

CWD forms of MTB have been linked to the aetiology of Crohn's disease, sarcoidosis and arthritis (Grange, 1992). However, it remains controversial.

Universal RT-PCR and species-specific MTB complex nested-PCR have detected live MTB organisms in the synovial tissue of early- and late-stage of rheumatoid arthritis (RA) patients (Kempsell, *et al.*, 2001). However, the presence of MTB in joint tissue did not appear to be associated with RA as MTB organisms were detected in a very small number of early-stage RA patients. An association of MTB with late-stage RA is difficult to establish due to possible opportunistic colonisation of already diseased and compromised tissue (Kempsell, *et al.*, 2000).

On the other hand, failure to detect MTB in latently infected people using any of the staining methods employed in this study, may have occurred due to the presence of a very few numbers of bacilli. Recently published work on a latent mouse model, which used a low infecting dose of bacilli also found it difficult to demonstrate bacilli in latently infected mice by immunohistochemical staining (Arriaga, *et al.*, 2002).

The frequent presence of positive labelling in the cytoplasm of cells suggests that these are likely to represent whole organisms. Naked DNA cannot persist in the cytoplasm as this is usually taken up by the nucleus immediately (Deretic, V. Personal communication). However, positive labelling has also been observed inside the nucleus of some cells, which suggests entry of free naked DNA into the nucleus. Micro-dissection and analysis of these positive labelled cells under the electron microscope should reveal the real nature of the bacilli, and whether or not they have retained their morphology.

## **Chapter 8**

### **8. Conclusions, speculations, and avenues for future investigations**

The tuberculin skin test is the method widely in use to screen latently infected people and to evaluate the efficacy of BCG vaccination (Stanford and Lema, 1983, Tuberculosis prevention trial 1979).

Enhancement of protection against TB by BCG in Sri Lanka cannot be calculated, as this study did not attempt to differentiate between Koch-like and Listeria-like responses. In addition, the number of unvaccinated children included in this study is not sufficient to calculate the efficacy of BCG at different study locations. However, the tuberculin skin test data of Sri Lankan school children has indirectly provided evidence of the influence of BCG vaccination on recognition of species-specific antigens (Chapter 3).

For the first time, reaction to skin test reagents was studied amongst the Tamil population in Jaffna, Sri Lanka. The exact epidemiological trend of tuberculosis in this area is not known due to the political situation. This part of Sri Lanka had close contact with South India until the early 1980's through travelling. In addition, it is believed Sri Lankan Tamils migrated from South India to the Northern part of Sri Lanka many years ago. Tuberculin skin test studies in South India have shown that BCG renders 0% protection against TB in this population (Tuberculosis prevention trial 1979). Vaccinated people showed Koch-type responses in this area. A large fraction of older children (about 66%) in Jaffna showed a reaction size of  $>10$  mm to the tuberculin skin test reagent (Table 3.5). If the sizes of positive tuberculin reactions are important, the high incidence of large-sized reactions amongst older children in Jaffna is worrying. This observation suggests that the immune response in these children is polarised towards the Th2 response, which indicates increased susceptibility to tuberculosis. Evaluation of the skin test responses according to 'Koch-like' and 'Listeria-like' reactions might address the above observation. At present, it is not

clear whether any common genetic factor in the South Indian and Jaffna populations is responsible for the observed phenomenon.

The role of vitamin D3 in the maturation and activation of human macrophages has been demonstrated by many studies e.g. (Rook, 1988)(Davies PDO, 1985) (Wilkinson, *et al.*, 2000). The high incidence of tuberculin positivity amongst unvaccinated children in Jaffna compared to children in Hambanthota may be an indication of possible association of vitamin D3 deficiency in Jaffna children, who are predominantly vegetarians with a high risk of susceptibility to TB (Chapter 3). This was the case in a predominantly vegetarian population of Gujarati Asians resident in west London. Those with serum vitamin D levels too low for detection had an almost tenfold increase in their risk of active tuberculosis (Wilkinson, *et al.*, 2000).

*In vitro* studies have demonstrated increased production, in the presence of 1,25-dihydroxy vitamin D3, of superoxide dismutase and nitric oxide synthase, which are the most important host defence mechanisms against intracellular pathogens (Rockett, *et al.*, 1998, Sly, *et al.*, 2001). Vitamin D3 facilitates accumulation of cytosolic oxidases (NADPH oxidase) responsible for the oxidative burst (Rockett, *et al.*, 1998, Sly, *et al.*, 2001). However, the exact role of Vitamin D at the cell biology level in the killing of intracellular MTB is not clear at present. Furthermore, a recent study has shown that the relationship between vitamin D and susceptibility to TB is affected by a human genetic factor. In India, a study of the wives of men with tuberculosis suggested that the homozygous TT vitamin D receptor (VDR) genotype was associated with susceptibility and the tt genotype with resistance to the disease (Selvaraj, *et al.*, 2000). Insight into the regulation of Vitamin D3-induced human monocyte antimycobacterial activity would be valuable in increasing our understanding of the pathogenesis of tuberculosis.

Failure to diagnose latent tuberculosis infection is the major obstacle to prevent reactivation of latent infection. None of the currently available methods can be used as a gold standard to evaluate a newly developed diagnostic method. The 16kDa antigen is valuable in the diagnosis of latently infected people (Chapter 4). The IgG oligoclonal response to this antigen in the elderly suggests that analysis of IgG isotypes, based on the current knowledge of the influence of T cell-

secreted cytokines on human immunoglobulin isotype expression (Sousa, *et al.*, 1998), and the T cell responses to this antigen, will be profitable.

Like other immunology-based assays that are in use to detect latent infection, the presence of an IgG oligoclonal response does not necessarily mean that the person currently harbours the bacilli (Orme, 2001). Evaluation of IgG and IgM oligoclonal responses during therapy of neurosyphilis has shown that successful treatment of the treponemal infection leads to the disappearance of oligoclonal IgM over 2-3 months, but persistence of the oligoclonal IgG continue for several years (Thompson and Keir, 1990). Therefore, evaluation of the IgM response to the 16kDa antigen in the elderly may be a reliable method to demonstrate the presence of tubercle bacilli during latent tuberculosis infection.

Even though several studies have detected a humoral response to the 16kDa antigen by ELISA in the CSF of TBM patients, only a polyclonal response was detected in the CSF of tuberculous meningitis patients in this study. However, intrathecal expansion of specific B cell clones against 38kDa antigen has proved its usefulness in the diagnosis of tuberculous meningitis (chapter 4).

For the first time, this study has detected MTB DNA in the sputum of elderly people who were not showing clinical symptoms characteristic of tuberculosis (chapter 5). However, genotyping of the DNA extracts revealed that there was no community transmission, suggesting that the bacilli shed are either very low in number or are in an inactive form, not virulent enough to cause a new infection. This observation raises many questions. Are all the latently infected people shedding the bacilli in their sputum? Are they living bacilli? Is there more than one form of tubercle bacilli during latent infection? If they are being shed in the sputum, are they slowly replicating in the lung?

An earlier study has shown late emergence of tubercle bacilli in culture from pulmonary lesions resected from humans (Hobby, *et al.*, 1954). The same study employed special processing procedures of specimens prior to culture. Adoption of the same sample processing technique and a long period of incubation of cultures would be valuable. Isolation of viable MTB from these elderly people

would be invaluable and would open up the mystery behind the latent tuberculosis infection.

One of the most important factors in the development of tuberculosis is the poorly understood ability of the tubercle bacillus to persist in the tissues for long periods. According to the accepted view of the pathogenesis of tuberculosis, it is widely believed that bacilli persisting during latent tuberculosis infection lurk in macrophages residing in the granulomas (Rook, 1994). However, the use of *in situ* PCR to detect DNA specific for MTB in tissues from different anatomical sites of people who died from causes other than TB has shown that this bacterium can lurk intracellularly without histological evidence of tuberculosis lesions (chapter 6 & 7). MTB DNA was detected not only in macrophages, but also in cells not normally regarded as phagocytic. From the present study it cannot be concluded whether the DNA detected represent the whole bacillus or just the fragments of MTB DNA. Free DNA does not usually exist in the cytoplasm; since it is immediately taken up into the nucleus (Deretic, V. Personal communication). Therefore, the DNA detected most probably represents whole bacilli persisting in the cytoplasm. A recent study using a latent mouse model has also demonstrated the presence of MTB DNA in histologically normal lung tissues of latently infected mice. In fact, in this study, MTB was isolated in culture from those tissues that harboured MTB DNA in histologically normal lung tissues (Arriaga, *et al.*, 2002). Detection of mRNA for the 85B antigen or isolation of bacilli on culture from human autopsy material would reveal the metabolic status of the bacilli. This could be done by performing necropsy as soon as possible after death of people from high TB incidence areas and immediately storing the specimens at -70°C.

According to the observations made in this study, postulates based on gradual oxygen withdrawal in latency, which thereby signals bacteria to change metabolically are irrelevant (Wayne, 1994, Wayne and Hayes, 1996). Autopsy data from the 1920s has also shown that tubercle bacilli in patients with quiescent tuberculosis may reside outside granulomas (Opie and Aronson, 1927). At present, it is not known why the bacilli in well-oxygenated tissues are not replicating. A study at the cellular level (organelle biogenesis) of survival

mechanisms of MTB in non-professional phagocytic cells would address this question.

No detection of acid-fast bacilli at the site of the detected DNA raises the possibility of non-acid-fast or CWD forms of bacilli during latent tuberculosis infection. Spoligotyping of DNA extracts from people who have died from causes other than TB has revealed an entirely different genotype from those found in clinically evident tuberculosis (chapter 7) and those found in the sputum of elderly subjects (chapter 5).

The presence of almost all the spacers in the DNA extracts from the people who died from causes other than TB makes it tempting to suggest that those bacilli causing latent infection may be an ancestral form. This is because changes (decrease) in the spacer number are unidirectional. In the living tissues, transposition of insertion elements may have caused changes in virulence, indicated by the presence of bacilli with a genotype similar to that of clinical isolates in the sputum of elderly people showing no clinical symptoms characteristic of TB. Therefore, latently infected people may contain a spectrum of bacilli, from truly dormant organisms to slowly replicating bacilli that may be the ones detected in the sputum of elderly people. Long-term protection conferred by isoniazide monotherapy in immunocompromised people supports the speculation that there may be a few slowly replicating bacilli in latently infected people. This view is further supported by the finding that protection ceases in HIV-positive persons at the end of tuberculosis chemoprophylaxis treatment with isoniazid (WHO, 2001).

Isolates of *M. bovis* and MTB, with an identical complete spoligotype pattern to the one found in DNA extracts from people who died from causes other than TB, have been found in the International database at RIVM. The use of these strains in animal models would be valuable in understanding the pathogenesis of latent tuberculosis. Furthermore, insight into the genetic and virulent nature of these strains may be helpful in understanding the nature of the bacilli during latent tuberculosis infection. If they are really less virulent and ancestral forms causing the latent infection, exploitation of these strains as vaccines may be profitable.

It is quite puzzling to see different spoligotype patterns in MTB DNA from the different anatomical sites of the same pulmonary TB patient, as it was believed all extra-pulmonary tuberculosis arise from the progeny of the bacilli that has been disseminated to these sites during the primary infection. In other words, those persisting at extra-pulmonary sites are different from the bacillus in the lung. This observation raises many questions. Have the bacilli, which disseminated from the lung adapted themselves to the different conditions which exist at different anatomical sites? Alternatively, are there organ-specific strains that can only colonise a particular organ? People living in high TB incidence areas may be infected with multiple strains and the bacilli that can adapt to a particular organ may colonise that organ. If bacilli persist at different anatomical sites, why does the imbalance in the immune system created during active pulmonary infection not cause reactivation at other anatomical sites? Is it because immune response against the active disease prevents the reactivation of the bacilli persisting in these other anatomical sites?

On the other hand, the observed difference in the spoligotype pattern may have occurred due to experimental factors. As discussed in chapter 5, PCR inhibitors and quality of the DNA from different anatomical site may have caused the observed difference. A recent case study has reported transmission of TB through donor organs (Graham, *et al.*, 2001). Identical strains were isolated from two transplant patients who received kidney and liver, respectively, from the same donor, suggesting dissemination of single strain to the distant organs.

In conclusion, the observations made in this study have opened up many avenues for future research. MTB is a resilient organism that can adapt to a wide range of environmental conditions. Tuberculosis can only be eradicated from this world with the clear understanding of pathogenesis of latent tuberculosis infection. This should eventually provide information to develop an efficient diagnostic method to detect latently infected people and to devise a strategy, vaccine or drug, to stop the development of latent tuberculosis infection or to prevent its reactivation.

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## Appendix 1

### Solutions for staining

#### 1. 2% Sodium hydroxide-N-acetyl-L-cysteine solution

NaOH	20g
Na <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> .2H <sub>2</sub> O	14.5g
Distilled water	1litre

#### 2. Phosphate buffer pH 6.8

KH <sub>2</sub> PO <sub>4</sub>	4.54g
Na <sub>2</sub> HPO <sub>4</sub>	4.73g
Distilled water	1 litre

#### 3. 1% acid alcohol

NaCl	25.0g
Distilled water	1250 ml
Methanol	3750 ml
Concentrated HCl	25 ml

Salt was added to water followed by alcohol and finally by concentrated HCl.

#### 4. 0.1% KMnO<sub>4</sub>

KMnO <sub>4</sub>	2.0g
Distilled water	2 litre

#### 5. Carbolfuchsin (Stock solution)

##### Solution A

Basic fuchsin (Sigma)	20.0g
Absolute alcohol	200 ml

##### Solution B

80% phenol	125 ml
Distilled water	1880 ml

Add solution A to B and dilute 1/20 in distilled water to use.

## 6. Malachite green

Malachite green	12.5g
Distilled water	2.5 litre

## LJ slopes for culture

### A. Mineral salt solution

KH <sub>2</sub> PO <sub>4</sub>	2.4g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.24g
Mg <sub>3</sub> (C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ) <sub>2</sub> .14H <sub>2</sub> O	0.6g
Asparagine	3.6g
Glycerol	12 ml
Distilled water	600 ml
2% Malachite green	20 ml

### B. Beaten egg

Nineteen eggs: Eggshell sterilised with methylated spirit and cracked with sterilised knife. Whisked egg was filtered through sterile sieve. Beaten eggs were mixed with mineral salt solution and distributed into sterile containers aseptically.

## Solution and reagents for ELISA

1. Purified recombinant proteins were obtained in powder form and suspended in phosphate saline buffer (PBS) and aliquots were stored at -20°C.

### 2. Coating buffer PBS pH 7.4

Sodium chloride (NaCl)	8.0g
Potassium dihydrogen orthophosphate (KH <sub>2</sub> PO <sub>4</sub> )	0.2g
Di-sodium hydrogen orthophosphate dihydrate (Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O)	1.135g
Potassium chloride (KCl)	0.2g
Non-pyrogenic water (Baxter)	1 litre

### 3. Washing buffer PBS/Polyoxy ethylene sorbitan monolaurate (Tween 20) (Sigma) pH 7.4

Sodium chloride (NaCl)	8.0g
Potassium dihydrogen orthophosphate (KH <sub>2</sub> PO <sub>4</sub> )	0.2g
Di-sodium hydrogen orthophosphate dihydrate (Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O)	1.135g

Potassium chloride (KCl)	0.2g
Non-pyrogenic water	1 litre
Tween 20 (Sigma)	0.5 ml

#### 4. Blocking buffer and diluent

PBS pH 7.4	1 litre
Tween 20	0.5 ml
Milk powder (Marvel-super market grade)	5 g

#### 5. Substrate 2,2-Azino-di-(3 ethyl benzthiazoline sulfonic acid) (ABTS)

50 mg ABTS in 100 ml citrate phosphate buffer (CPB)

#### 6. Citrate phosphate buffer (CPB)

##### Solution A:

Citric acid	21.01g
Ultra pure water	250 ml

##### Solution B:

Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	17.79g
Ultra pure water	250 ml

Prepared by adding solution A to B to reach pH 4.1

#### 7. Reaction stopping solution

Sodium fluoride (NaF) (Sigma)	96 mg
Ultra pure water	50 ml

### **Solutions for simple immunoblotting assay**

#### 1. Blocking solution

Sodium chloride (NaCl) (Sigma)	9.0g
Milk powder (Marvel-super market grade)	2 g
Non-pyrogenic water (supplier)	100 ml

**2. Sodium acetate buffer pH 5.1**

Sodium acetate (Merck)	27.5g
Distilled water	1 litre

**3. Colour reagent**

4-chloro-1-naphthol (Sigma)	50mg
Methanol	20 ml
Sodium acetate buffer	10 ml
Distilled water	90 ml
30% (w/w) H <sub>2</sub> O <sub>2</sub> (Sigma)	120 µl

**Solutions and reagents for IEF**

**1. Aqueous glycerol**

Glycerol (Merck, U.K.)	10ml
Distilled water	90ml

**2. 0.9% Saline (Normal saline)**

Sodium chloride (NaCl)	9.0g
Non-pyrogenic water (supplier)	1 litre

**3. Blocking solution**

0.9% saline	100 ml
Milk powder (Marvel-super market grade)	2 g

**3. Cathodic electrode: Sodium hydroxide 1M**

Sodium hydroxide (NaOH) (Merck)	40g
Distilled water	1 litre

**4. Anodic electrode: Sulphuric acid 0.05M**

Concentrated Sulphuric acid (H <sub>2</sub> SO <sub>4</sub> ) (Merck)	1.4ml
Distilled water	500ml

5. 0.02M Sodium acetate buffer pH 5.1

Sodium acetate (Merck )	27.5g
Distilled water	1litre

**Solution for immunohistochemistry**

1. Phosphate buffer pH 7.6

NaH <sub>2</sub> PO <sub>4</sub> . 2H <sub>2</sub> O	57.7 g
NaH <sub>2</sub> PO <sub>4</sub>	12.9 g
Distilled water	1 litre

2. PBS

Phosphate buffer	100 ml
Saline 0.9%	900 ml

3. Blocking buffer

PBS	100 ml
BSA (Sigma)	3 g

4. DAB (Diamino benzidine )

DAB (Sigma)	1 mg
PBS	1 ml
30% (w/w) H <sub>2</sub> O <sub>2</sub>	40 µl

**Solutions for PCR assay**

1. Borate buffered saline pH 8.0

Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> .10H <sub>2</sub> O (Sodium tetraborate)	3.63g
H <sub>3</sub> BO <sub>3</sub> (Boric acid)	5.25g
NaCl	6.19g
Tween 80	5.0µl
Ultra pure water	1 litre

Autoclaved and stored at RT

## 2. Demineralisation solution

Proteinase K (20 mg/ml) (Finnzymes)	240 µl
0.5M EDTA pH 8.0 (Promega)	4.56 ml

## 3. 0.1M Tris-HCl buffer

Trizma hydrochloride (Sigma)	15.76 g
Molecular biology grade water	1 litre
pH adjusted to 6.4	

## 4. GuSCN/Tris-HCl buffer/ L2 wash buffer

GuSCN (Sigma)	11.82 g
0.1M Tris-HCl buffer pH 6.4	10 ml

## 5. L6 lysis buffer

GuSCN/Tris-HCl buffer pH 6.4	10 ml
0.2 M EDTA pH 8.0 (Promega)	1 ml
Triton X 100 molecular grade (Sigma)	0.13 ml

## 6. Preparation of silica suspension

100 g silica oxide (sigma) was added to 500 ml of ultra pure water, left overnight, the supernatant was discarded and another 500 ml of water was added and left overnight. The supernatant was discarded again and silica was re-suspended in a small volume of water and stored in 40 µl aliquots at room temperature.

## 7. X10 Tris Borate-EDTA (TBE) buffer

Trizma base (Sigma)	53.5 g
EDTA (Sigma)	3.7 g
Boric acid (Sigma)	27.5 g

Volume 300 ml of ultra pure water was heated to 50°C and above chemicals were dissolved using a magnetic stirrer. Ultra pure water was added to this to give final volume of 500 ml.

#### 8. Electrophoresis buffer

Ultra pure water	315 ml
X10 TBE	35 ml
Ethidium bromide (10mg/ml)(Sigma)	5 $\mu$ l

#### 9. Gel

Nusieve agar (Flowgen)	1.5 g
Ultra pure water	45 ml
X10 TBE	5 ml
Ethidium bromide	2 $\mu$ l

Agar was dissolved in ultra pure water by heating in a microwave for 10 minutes. After cooling it down TBE and ethidium bromide were added and poured into a gel tray. Gel was allowed to set for 45-60 minutes prior to electrophoresis.

### **Solutions for spoligotyping**

#### 1. 0.5 M EDTA, pH 8.0

EDTA (Sigma)	186.12g
Distilled water	1litre

#### 2. 20 mM EDTA, pH 8.0

Dilute 0.5 M EDTA 25 times

#### 3. 20xSSPE

Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	35.6g/l
3.6 M NaCl	210.24g/l
20mM EDTA	7.4g/l

The ph should be 7.4. Autoclaved and stored at room temperature for no longer than one year.

#### 4. 2xSSPE

Dilute 20 x SSPE ten times with distilled water.

5. 10% SDS

10g SDS (Merck) per 100 ml distilled water.

6. 2xSSPE/0.1%SDS

Add 100 ml 20xSSPE and 10 ml 10% SDS to 890 ml distilled water.

7. 2xSSPE/0.5%SDS

Add 100 ml 20xSSPE and 50 ml 10% SDS to 850 ml distilled water.

**Solutions for *in situ* PCR**

1. 0.02M HCl

Concentrated HCl                    30.6 µl

Distilled water                        50 ml

2. X1PBS pH 7.35

KCl                                    0.2g

K<sub>2</sub>HPO<sub>4</sub>                            0.12g

NaCl                                    8.0g

Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O                    2.16g

Distilled water                        1 litre

Autoclaved and kept at room temperature

3. 0.01% Triton X-100

Triton X-100                        50 µl

PBS pH 7.35                        50 ml

4. Proteinase K (1µg/ml)

Proteinase K (20mg/ml) (Finnzyme)    5 µl

PBS pH 7.35                        100 ml

5. 20% Acetic acid

Acetic acid                            20 ml

Sterile ultra pure water            80 ml

Solutions 1,3,5 were filter sterilised using 0.45 µm Acrodisc® syringe Filters (Pall Gelman Laboratory).

6. TBS pH 7.6 for washing

Trizma-base (50 mM)	0.61g
NaCl (150 mM)	0.88g
Distilled water	100 ml

7. TBS-MgCl<sub>2</sub> (substrate diluent) pH 9.5

Trizma-base (100mM)	1.22g
NaCl (50mM)	0.29g
MgCl <sub>2</sub> (50mM)	0.47g
Distilled water	100 ml

8. TBS for antibody dilution pH 7.5

Trizma-base (100mM)	1.22g
NaCl (150mM)	0.88g
Distilled water	100 ml

## Appendix 2

### Names and address of suppliers

**Sigma Aldrich**, Fancy Road, Poole, Dorset BH 12 4QH, UK

**Finnzymes**, P.O.Box 148, Finland 02201

**DAKO**, Angel Drove, Ely, Cambridgeshire CB7 4ET, UK

**Lionex Diagnostics and therapeutics**, Mascheroder, Weg 1b D-38124, Braunschweig, Germany

**Oswel DNA service**, LAB 505, Medical & Biological Sciences building

University of Southampton, Boldrewood, Bassett Crescent East, Southampton SO16 7PX, UK

**FMC Bioproducts**, Flowgen, Novara House, Excelsior Road, Ashby Park, Ashby de la Zouch, Leicestershire LE 65 1NG, UK

**Aersham Pahrmacia Biotech UK limited**, Amersham Place, Little Chalfont, Bucks HP7 9NA, UK

**Boehringer Mannheim**, Bell Lane, Lewes, East Sussex BN7 1LG, UK

**Applied Biosystems**, PE United Kingdom, Kelvin Close, Birchwood Science Park North, Warrington, Cheshire WA3 7PB, UK

**Merck Ltd (BDH)**, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN, UK

**Quiagen Ltd**, Boundary Court, Gatwick Road, Crawley, West Sussex RH10 9AX, UK

**Stratech Scientific Ltd**, 61-63 Dudley street, Luton, Bedfordshire LU2 0NP, UK

**Fisher Scientific UK Ltd**, Bishop meadow road, Loughborough, Leicestershire LE11 5RG

**Pall Corporation**, Europa house, Havant street-Portsmouth, Hampshire, England PO1 3PD

**Whatman plc**, Whatman house, St Leonard's road, 20/20 Maidstone, Kent ME16 0LS, UK

**Ultra-Violet Products Ltd**, Unit 1, Trinity hall estate, Nuffield road, Cambridge CB4 1TG, UK

**Scientific Laboratory Supplies Ltd**, Wilford industrial estate, Ruddington lane, Wilford, Nottingham NG11 7EP

**Helena Biosciences**, Colima avenue, Sunderland enterprise park, Sunderland, Tyne & Wear, SR5 3XB, UK

**Promega UK**, Delta house, Chilworth science park, Southampton SO16 7NS, UK

**The Microbioiogical Supply Company**, P.O.Box 23, Toddington, Beds LU5 6DW

**Baxter Health Care Ltd**, Wallingford road, Compton, Nr. Newbury, Berkshire RG20 7QW

# Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection

R Hernández-Pando, M Jeyanathan, G Mengistu, D Aguilar, H Orozco, M Harboe, G A W Rook, G Bjune

## Summary

**Background** A third of the world's population has latent infection with *Mycobacterium tuberculosis*, and in areas of low endemicity, most cases of active tuberculosis arise as a result of reactivation of latent bacilli. We sought to establish the cellular location of these latent organisms to facilitate their elimination.

**Methods** We applied in-situ PCR to sections of macroscopically normal lung tissue from 13 individuals from Ethiopia and 34 from Mexico who had died from causes other than tuberculosis. Sections of lung tissue from six Norwegian individuals (ie, individuals from a non-endemic population) acted as negative controls, and six Ethiopian tuberculosis cases acted as positive controls.

**Findings** Control necropsy samples from the Norwegian individuals were all negative by in-situ PCR and conventional PCR, whereas all samples from known Ethiopian tuberculosis cases were positive by both methods. However, in macroscopically normal lung tissue from Ethiopian and Mexican individuals without tuberculous lesions, the in-situ PCR revealed five of 13 and ten of 34 positive individuals, respectively. These results were confirmed by conventional PCR with extracted DNA. Positive cells included alveolar and interstitial macrophages, type II pneumocytes, endothelial cells, and fibroblasts.

**Interpretation** *M tuberculosis* can persist intracellularly in lung tissue without histological evidence of tuberculous lesions. *M tuberculosis* DNA is situated not only in macrophages but also in other non-professional phagocytic cells. These findings contradict the dominant view that latent organisms exist in old classic tuberculous lesions, and have important implications for strategies aimed at the elimination of latent and persistent bacilli.

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See Commentary page 2113

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## Introduction

About a third of the world's population is infected with *Mycobacterium tuberculosis*.<sup>1</sup> In areas of high endemicity, the first mycobacterial infection usually occurs in childhood. In most individuals, it is kept under control by the immune system, and only in about 10% does it lead to disease.<sup>2</sup> However, in primary tuberculosis, even when successfully controlled by the immune system, not all bacteria are eliminated. Some bacilli remain in the tissues in a latent state for the rest of the individual's life.<sup>3</sup> This latent state is important for two reasons.

First, in countries with low or moderate tuberculosis endemicity, most cases of tuberculosis result from reactivation of latent infection.<sup>4–6</sup> Second, the persistence of organisms that are resistant to chemotherapeutic agents requires prolonged chemotherapy of active disease, which leads to problems in terms of logistics and compliance.<sup>7</sup> These persistent bacteria could exist in a physiological state similar to that of latent bacteria. Thus, new strategies for the eradication of latent organisms are required, and such strategies will arise from a better understanding of the mechanism by which the tubercle bacilli persist.

Experimental work in laboratory animals has suggested that alveolar macrophages are the first cells infected by *M tuberculosis*.<sup>8</sup> These infected macrophages are then surrounded by newly recruited macrophages and lymphocytes that form the characteristic tuberculous granuloma.<sup>9</sup> Bacterial latency is assumed to occur in these classic tuberculous lesions. However, this assumption does not explain the facts of reactivation tuberculosis—ie, that the primary lesion can occur anywhere in the lung, yet more than 90% of progressive cavitatory “post-primary” disease occurs in the upper lobes.<sup>10</sup> Moreover, in 1927, Opie and Aronson reported that fewer than 10% of old lesions contained live bacilli, whereas such organisms could be recovered from macroscopically normal lung tissue in almost 50% of people who had died from causes other than tuberculosis.<sup>11</sup>

The aims of this study were to determine whether *M tuberculosis* DNA is indeed present in lung tissue with no specific histopathology, and if so, to identify its cellular location.

## Methods

### Patients

13 individuals who had died from violent causes (seven), acute infection (two), heart attack (one), drowning (two) or starvation (one) were selected from the Department of Pathology at the Menelik II Hospital in Addis Ababa, Ethiopia. Individuals who were positive for HIV-1 by the Welcozyme ELISA were excluded. Tuberculin status was unknown for all individuals. The study was approved as ethical by the Ethiopian Science and Technology Commission.

*Preparation of lung tissue*

As soon as possible after death (mean 24 h, range 6–36 h), 3–5 cm diameter blocks of macroscopically normal lung tissue were obtained from the right upper or left upper lobes, and immediately fixed in 10% formaldehyde dissolved in phosphate-buffered saline. After fixation, the lung tissue was dehydrated in increasing concentrations of ethyl alcohol, and embedded in paraffin. For histological examination, 5 µm sections were cut and stained with haematoxylin and eosin and Ziehl-Neelsen stains. These paraffin blocks were also used for mycobacterial DNA localisation by in-situ PCR. In addition, smaller fragments of lung tissue were obtained from each patient and immediately frozen by immersion in liquid nitrogen. These samples were used for mycobacterial culture and direct microscopic assessment for acid-fast bacilli. Lung tissue obtained at necropsy from six Ethiopian individuals who died from pulmonary tuberculosis were processed in the same way and used as positive controls. Three of these tuberculosis cases were HIV-1-positive.

Paraffin-embedded blocks of lung tissue obtained from 34 individuals at necropsy were also selected from the Department of Pathology files at the Instituto Nacional de la Nutricion and Hospital Infantil in Mexico City, Mexico. Individuals were HIV-1-negative, of unknown tuberculin status, and had died from causes other than tuberculosis. The tissue sampled was from the hilar region or from the apex near to the pleura.

Six paraffin blocks of lung tissue obtained at necropsies in the Department of Pathology, Haukeland University Hospital, Bergen, Norway were used as negative controls. Annual tuberculin surveys have shown that community transmission of tuberculosis has been totally interrupted in Norway since the 1950s (Norwegian National Tuberculosis Register, Oslo).

*Detection of mycobacterial DNA by in-situ PCR*

Coded lung sections were mounted on silane-coated slides, deparaffinised for 18 h at 60°C, and sequentially immersed in xylene (30 min at 37°C), absolute ethanol, 75% ethanol, 50% ethanol, 25% ethanol, and water. Cells were made permeable by incubation at room temperature in 0.02 mol/L HCl for 10 min, followed by 0.01% triton X-100 for 90 s. Proteins were depleted by incubation with 1 mg/L proteinase K (Gibco, Paisley, UK) for 30 min at 37°C. The proteinase was then inactivated by boiling in a microwave for 15 s, and the section was plunged immediately into 20% acetic acid for 15 s to inactivate endogenous alkaline phosphatase.

PCR was done by incubation of the sections with 50 µL 1X reaction buffer (Gibco, BRL), 1.5 U Taq polymerase, 2 mmol/L MgCl<sub>2</sub>, 40 µmol/L dNTP, 0.2 µmol/L dUTP labelled with digoxigenin (Boehringer Mannheim, Lewes, UK), and 60 pg each of IS6110 *M. tuberculosis* insertion sequence primers. The sequences of the primers were: 5'CCT GCG AGC GTA GGC GTC GG 3' (sense) and 5'CTC GTC CAG CGC CGC TTC GG 3' (antisense). The slides were sealed with the Assembly tool (Perkin Elmer, Cambridge, UK) and placed in a Touch Down thermocycler (Hybaid, Ashford, UK). Amplification comprised denaturation at 95°C for 1 min, annealing at 70°C for 1 min, and extension at 72°C for 1 min, for 35 cycles.

PCR products were detected with alkaline-phosphatase-conjugated sheep antibodies against anti-digoxigenin (Boehringer Mannheim) diluted 1/500. The chromogen was 5-bromo-4-chloro-3-indolyl

phosphate toluidine salt and tetrazolium nitroblue (Boehringer Mannheim) diluted 1/50. Sections were counterstained with nuclear fast red to avoid any interference with the blue signal generated by mycobacterial DNA in the in-situ PCR. To avoid bias, one section from one block from each individual was used in masked assays and it was scored positive if the blue reaction product was seen.

Lung sections from six patients with tuberculosis were used as positive controls. The negative controls were samples of non-infected mouse lung and lung tissue from patients identified as being negative for *M. tuberculosis* DNA in preliminary experiments. To control for false positives due to DNA repair, duplicates of each test section were subjected to PCR without the Taq enzyme.

*Detection of mycobacterial DNA by conventional PCR*

Conventional PCR was done with the same primers, according to a protocol normally used on archaeological specimens where extraction and amplification of mycobacterial DNA are particularly difficult.<sup>12</sup> DNA extraction and liquid-phase PCR was done twice on each block on consecutive sections.

**Results**

Table 1 shows the data for the 13 Ethiopian samples tested by conventional and in-situ PCR protocols. Five were positive for mycobacterial DNA by in-situ PCR, and five by conventional PCR. Four of these five were positive by both methods. One sample was positive only by in-situ PCR, and another was positive only by conventional PCR. Thus 11 of 13 were concordant by the two methods.

Table 2 shows the data from the 34 Mexican individuals. Ten individuals were positive by in-situ PCR, eight of whom were also positive by conventional PCR. 12 of 34 were positive by conventional PCR, which was not significantly different from the rate of positivity in the Ethiopian samples. Two were positive only by in-situ PCR, and four were positive only by conventional PCR. Thus 26 of 34 were concordant.

Concordance was 100% for the control samples. None of the lung sections from the six Norwegian individuals showed positive labelling by either in-situ or

Patients' characteristics				Presence of mycobacterial DNA	
Sex	Evidence of clinical disease	Age (years)	Cause of death	In-situ PCR	Conventional PCR
Female	No	30	Accident	—	—
Male	No	35	Accident	—	+
Male	No	31	Heart attack	+	—
Male	No	30	Car accident	—	—
Male	No	27	Accident	—	—
Male	No	32	Drowning	+	+
Male	No	30	Bronchopneumonia	+	+
Male	No	35	Starvation	+	+
Male	No	40	Drowning	+	+
Female	No	25	Septic abortion	—	—
Male	No	43	Car accident	—	—
Female	No	15	Accident	—	—
Male	No	35	Accident	—	—
Female	Yes	40	Tuberculosis	+	+
Male	Yes	25	Tuberculosis	+	+
Male	Yes	30	Tuberculosis	+	+
Female	Yes	30	Tuberculosis	+	+
Male	Yes	40	Tuberculosis	+	+
Male	Yes	27	Tuberculosis	+	+

Table 1: Detection of mycobacterial DNA in lung tissue from Ethiopian necropsy samples

conventional PCR (table 2), and the six known tuberculosis cases were all positive by both methods (table 1).

None of the Ethiopian samples from individuals without tuberculous disease showed fibrotic nodules, scars, or calcifications suggestive of tuberculosis, and all 13 samples were negative for acid-fast bacilli by direct microscopy and by mycobacterial culture. By contrast, all of the six positive controls who died of tuberculosis showed acid-fast bacilli by direct microscopy and were culture-positive for *M tuberculosis*.

None of the Mexican individuals had shown signs or symptoms of tuberculosis before death, and none of them had shown lesions suggestive of tuberculosis in the lungs at necropsy. Similarly, there were no histopathological signs of tuberculosis in the tissue samples, despite the fact that 70% of the PCR-positive cases had died as a consequence of diseases which can cause secondary immunodeficiency (cancer, starvation, autoimmunity), and thus had an increased risk of acquiring tuberculosis. A higher rate of PCR positives was found among necropsy samples from the paediatric

hospital than among samples from adults. This is probably because the children's hospital recruits its patients from a lower socioeconomic stratum, and thus from a population with a higher risk of infection.

Despite the absence of tuberculosis, histological examination showed that neither the Ethiopian nor the Mexican tissues assessed were entirely normal. In every sample, lung tissue contained accumulations of carbon particles in the cytoplasm of interstitial or alveolar macrophages (figure), or in fibrous tissue near to the bronchial or vascular walls or more commonly in the subpleural space (figure).

In the Mexican tissue samples, small patches of bronchopneumonia and oedema were commonly present. In the Ethiopian samples, and in some Mexican individuals, fibrous tissue replaced the alveoli, was situated immediately below the apical pleura, and commonly extended in wedge-shaped areas into the lung substance. In some areas, this fibrous tissue was quite acellular.

An important aspect of these data is the cellular location of the positive signal seen by in-situ PCR in individuals who did not have tuberculous disease. Alveolar and interstitial macrophages were the most common cells with positive mycobacterial DNA labelling (figure). Round cells located on the alveolar surface were also positive. Their morphology and location indicated that these cells were type II pneumocytes (figure). Some endothelial cells and occasional fibroblasts in venules and intermediate-sized veins were also positive (figure). Subpleural fibrotic areas showed positivity in macrophages and fibroblasts (figure). Positive cells tended to be grouped together in foci of very variable size, whereas other parts of the same sections were entirely negative.

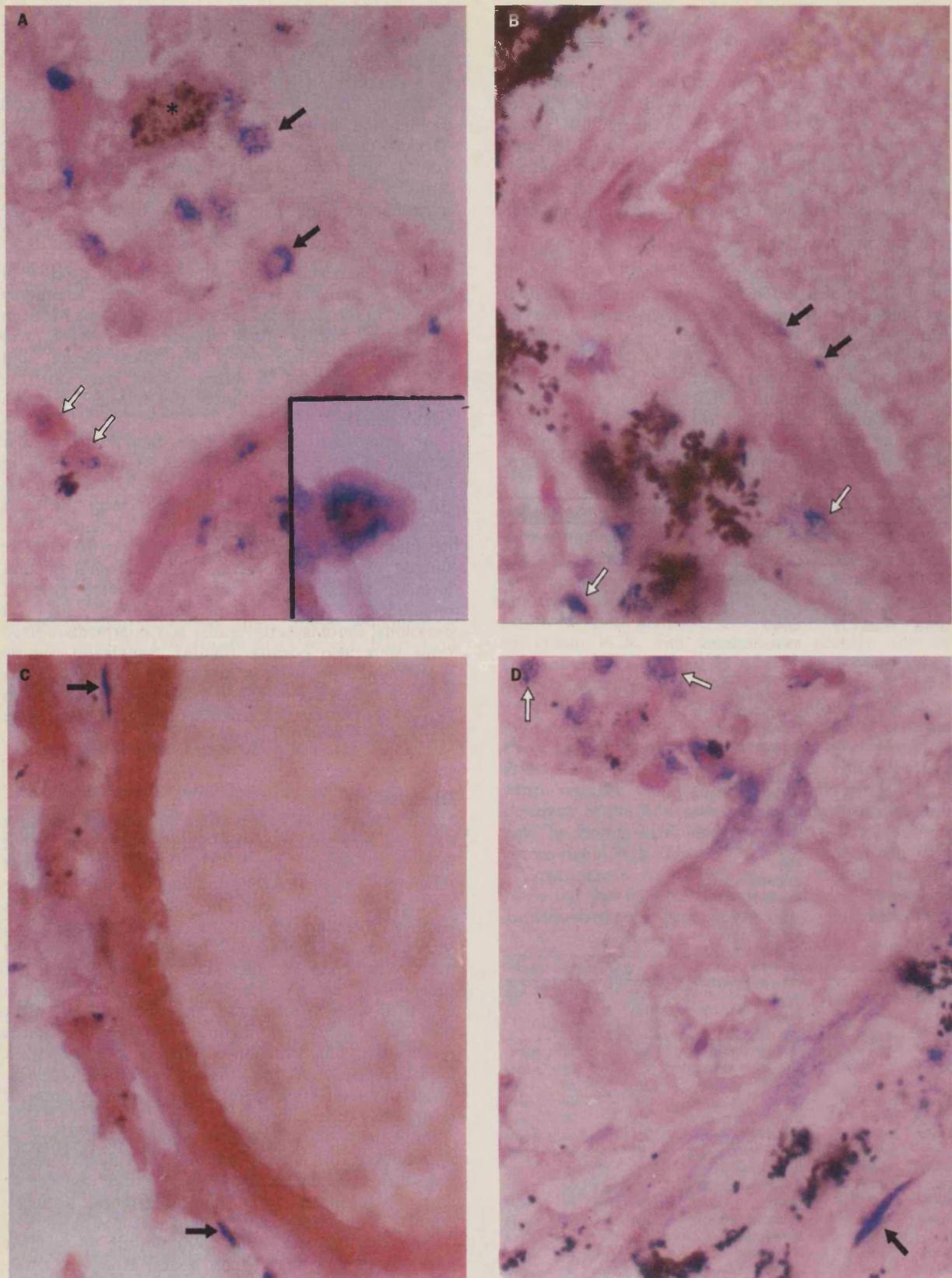
## Discussion

Latent tuberculosis occurs after an immune response has been generated to control the pathogen and force it into a quiescent state.<sup>13</sup> The diagnosis of latent tuberculosis is established, although not with absolute certainty, by the absence of clinical disease and a positive tuberculin skin test or a chest radiograph which shows scars or calcified nodules indicative of resolved primary tuberculosis infection.<sup>13</sup> People with latent tuberculosis do not transmit the disease, but in countries where tuberculosis endemicity is not high, they have a 2–23% lifetime risk of tuberculosis reactivation. This situation is different in areas with a high incidence of this disease: in these regions, exogenous reinfection seems to be the major cause of postprimary tuberculosis.<sup>5,6</sup>

Surprisingly little is known about the location of *M tuberculosis* in latently infected individuals.<sup>13</sup> Many years ago, Opie and Aronson<sup>11</sup> and Griffith<sup>14</sup> transferred lung homogenates from non-tuberculous necropsy samples to guineapigs, and showed that it is possible to recover live tubercle bacilli from fibrotic or calcified lung lesions and hilar lymph nodes. However, tissue fragments obtained from the apices or bases of macroscopically normal lung tissue yielded a higher percentage of positive samples than the diseased tissue.<sup>11</sup> This observation dating from 1927 is rarely cited. However, it was one of the pieces of evidence that led Balasubramanian and colleagues to propose that, rather than lying dormant in the primary complex, "the virulent bacilli lie dormant in a metastatic site seeded haematogenously within the vulnerable region".<sup>10</sup>

Patients' characteristics			Presence of mycobacterial DNA		
Country	Sex	Age (years)	Cause of death	In-situ PCR	Conventional PCR
Mexico	Female	12	Myeloblastic leukaemia	+	+
Mexico	Male	13	Cerebral palsy	+	-
Mexico	Female	16	Lupus erythematosus	-	-
Mexico	Female	17	Alveolar rhabdomyosarcoma	+	+
Mexico	Female	22	Gastric	-	-
Mexico	Male	26	Minimal change glomerulopathy	-	-
Mexico	Male	30	Liver cirrhosis	-	-
Mexico	Female	23	Lupus erythematosus	-	-
Mexico	Male	35	Diabetes mellitus type 1	-	-
Mexico	Male	6	Cardiac malformation	-	-
Mexico	Male	12	Cardiac malformation	+	+
Mexico	Female	31	Massive liver necrosis	-	-
Mexico	Female	36	Lupus erythematosus	-	-
Mexico	Female	45	Diabetes mellitus type 2	-	-
Mexico	Female	49	Myocardial infarction	-	-
Mexico	Male	46	Liver cirrhosis	-	-
Mexico	Female	15	Lymphoblastic leukaemia	+	+
Mexico	Male	15	Lymphoblastic leukaemia	+	+
Mexico	Male	14	Lupus erythematosus	+	+
Mexico	Male	52	Myeloma	-	-
Mexico	Female	58	Stroke	-	-
Mexico	Female	58	Liver cirrhosis	+	+
Mexico	Male	51	Acute pancreatitis	-	-
Mexico	Female	42	Renal failure and diabetes	-	-
Mexico	Female	55	Sepsis	-	-
Mexico	Female	58	Liver cirrhosis	-	+
Mexico	Female	57	Liver cirrhosis	-	-
Mexico	Male	51	Liver cirrhosis	-	+
Mexico	Female	56	Myeloid leukaemia	-	-
Mexico	Female	50	Phaeochromocytoma	-	+
Mexico	Male	48	Sepsis	-	+
Mexico	Male	54	Complications of spinal-cord surgery	+	-
Mexico	Female	37	Starvation	+	+
Mexico	Female	30	Hepatic necrosis	-	-
Norway	Male	38	Hodgkin's disease	-	-
Norway	Female	52	Carcinoma of the cervix	-	-
Norway	Female	36	Glioblastoma	-	-
Norway	Female	49	Melanoma	-	-
Norway	Female	45	Multiple sclerosis	-	-
Norway	Male	50	Carcinomatosis in pericardium	-	-

Table 2: Mycobacterial DNA in lung tissue from Mexican and Norwegian necropsy samples



**Representative micrographs of the cellular localisation of mycobacterial DNA revealed by *in-situ* PCR in lung tissue from a single donor without tuberculous disease**

A=Alveolar macrophages showing positive (blue) mycobacterial DNA labelling (black arrows). These cells are intermixed with other macrophages containing black phagocytosed carbon material (\*). Positive round cells on the alveolar wall correspond to type II pneumocytes (white arrows and insert). B=Vein endothelial cells (black arrows) and macrophages (white arrows) surrounding carbon deposits showing mycobacterial DNA labelling. C=Positive fibroblasts (black arrows) located in the adventitial layer from an intermediate-sized vein. D=A fibroblast (black arrow) and macrophages (white arrows) in fibrotic subpleural bands show strong mycobacterial DNA labelling. Original magnification  $\times 400$  (insert  $\times 100$ ).

In the guinea pig model used by these workers, this phase of haematogenous dissemination from the primary lesion is manifested as small tubercles, because growth of the disseminated bacteria occurs, and does not stop until about  $10^3$  colony-forming units and a tubercle have developed. Our results reveal that, in human beings, the DNA of *M. tuberculosis* is found in the absence of a histological reaction, suggesting that growth of the seeded bacteria might not occur unless there is reactivation of disease. The same has recently been shown in a murine model of latent tuberculosis in which it is possible to culture mycobacteria from lung homogenates in the complete absence of specific histopathology.<sup>15</sup> In human lung tissue and in latent mycobacterial infection in mice, microscopic examination usually fails to reveal acid-fast bacilli, so it is necessary to use molecular methods with high sensitivity like in-situ PCR for the detection of mycobacterial DNA.

We selected the IS6110 insertion sequence for the detection of mycobacterial DNA because PCR assays based on the amplification of this multicopy sequence can be specific and sensitive for the diagnosis of tuberculosis, with positive results in 95–100% of clinical samples from patients with cultures positive for *M. tuberculosis*.<sup>16</sup> This technique has allowed us to confirm and expand the results of Opie and Aronson.<sup>11</sup> Analysis of macroscopically normal lung tissues obtained at necropsy from individuals who lived in areas of high tuberculosis endemicity and who died from causes other than tuberculosis showed mycobacterial DNA in 38% and 29% of the samples from Ethiopia and Mexico, respectively by in-situ PCR, and in 38% and 35%, respectively by conventional PCR. There was a 79% overall concordance between the conventional and in-situ PCR results.

The positive signal that denoted the presence of mycobacterial DNA was seen in several types of cell. Alveolar and interstitial macrophages were the most common positive cells, but positivity was also seen in type II pneumocytes, endothelial cells, and fibroblasts, and particularly in cells located in or close to the subpleural fibrotic bands with variable deposition of carbon particles.

Several species of pathogenic mycobacteria including *M. avium*, *M. marinum*, and *M. tuberculosis*, have been shown to invade and replicate within a variety of non-professional phagocytes, such as fibroblasts, endothelium, and human epithelial cells.<sup>17–22</sup> It has always seemed paradoxical that *M. leprae* is routinely seen inside a variety of cell types in vivo,<sup>23</sup> whereas *M. tuberculosis*, which readily invades such cells in vitro, has been said to be exclusively extracellular or within macrophages and macrophage-derived cell types in vivo. It may be that the organisms in classic tuberculosis lesions are indeed confined to macrophages, because non-professional phagocytes that take up *M. tuberculosis* in vitro become exquisitely sensitive to the toxic effects of tumour necrosis factor  $\alpha$ , which is abundant in such sites.<sup>24,25</sup> However, the results presented here indicate that in vivo, non-professional phagocytic cells located well away from the active lesions could provide important sanctuaries with minimal antigen-presenting ability. The sites where we have shown *M. tuberculosis* DNA are clearly not anaerobic—a finding that agrees with the recent observation that metronidazole has no effect in the Cornell model of latent tuberculosis.<sup>26</sup>

These insights into the location of *M. tuberculosis*

DNA, indicating the likely location of latent bacteria, could accelerate the search for strategies for their elimination.

#### Contributors

Rogelio Hernandez-Pando, Gunnar Bjune, Gennene Mengistu, and Morten Harboe contributed to the background work, to the design of the study, and to the collection of clinical material. Mangalakumari Jeyanathan, Diana Aguilar, Gennene Mengistu, and Hector Orozco contributed to the design of the in-situ PCR protocol, and the assays shown were done by Mangalakumari Jeyanathan, Diana Aguilar, and Gennene Mengistu. Rogelio Hernandez-Pando did the histopathological analysis, and Graham Rook wrote the paper.

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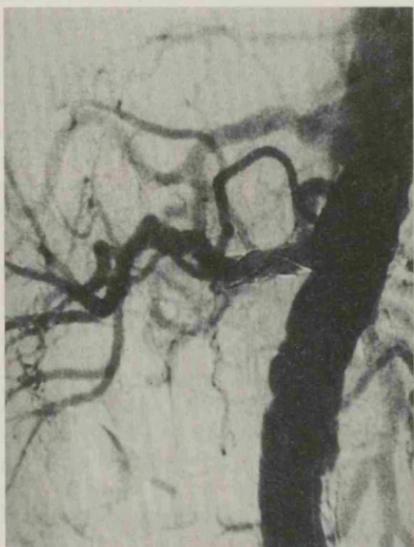
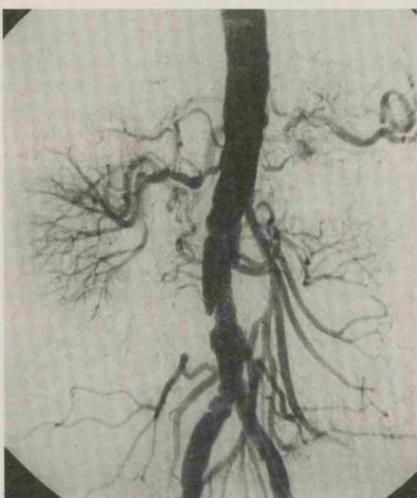
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## Clinical picture: Ipsilateral atherosclerotic and fibromuscular renal artery stenosis

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A 71-year-old lady was referred with drug-resistant hypertension. Aortography demonstrated diffuse aortic disease with significant atheromatous stenoses involving both proximal renal arteries. Selective injection on the right side showed typical appearances of fibromuscular dysplasia (FMD) in the distal main renal artery. The proximal stenosis was stented to 6 mm. Subsequently, it was discovered that she had been diagnosed with FMD 33 years previously.

The combination of atheromatous and fibromuscular renal artery stenosis in the same renal artery is very rare. It is well established that atherosclerotic RAS is a progressive disease and that severe stenoses progress to occlusion. In this case, it is hoped that stenting will delay any further deterioration in renal function.

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