Molecular and Immunological Studies on "Fully Treated" Long-Term Leprosy Patients

by

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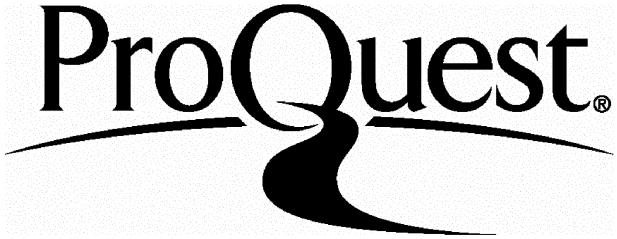

ABSTRACT

In a preceding visit, 279 leprosy patients in the Baba Baghi Leprosy Sanatorium in Iran with long histories of treatment with dapsone, were subjected to skin-testing with 4 new tuberculins, and randomised to receive an injection of saline as placebo or killed *M. vaccae* as immunotherapy.

A year later, in the first sampling visit, the study began on selected groups of these patients, each of whom provided sputum and skin-tissue fluid specimens for DNA studies, and serum samples for antibody estimation. Eighteen months later, in the second sampling visit, further samples were obtained from 50 of the same patients. The aim was to search for leprosy and tubercle bacilli by PCR in appropriate specimens, and relate the findings to the skin test, immunotherapy data, and immunoassays.

Three different DNA extraction methods for preparation of template DNA were evaluated. The chosen DNA extraction method was based on the lysing and nuclease-inactivating properties of guanidine thiocyanate (GuSCN) together with the nucleic acid-binding properties of diatoms. Species-specific primers for a 530-bp fragment of the gene encoding the 36 kDa antigen of *M. leprae* and for a 123-bp fragment of the repetitive DNA sequence (IS*6110*) of *M. tuberculosis* were used for the study and PCR results were compared with conventional methods. Using the chosen techniques, 25% of the selected "fully treated" long-term leprosy patients were found to be PCR-positive for *M. leprae* and 13.6% of the same patients were found to be PCR-positive for *M. tuberculosis*. Comparison of PCR results showed no concurrent positivity for both organisms.

The serum samples from the same selected patients were tested by

indirect ELISA to determine the level of IgG against seven mycobacterial antigen preparations. A modified technique was used to measure the level of agalactosyl IgG (G_0). The serological results correlated very well with the molecular findings, 81.1% of the leprosy PCR-positive patients could be confirmed with PGL-I ELISA and 83.3% of the tuberculosis PCR-positive patients were confirmed by the G_0 assay.

Additionally, 29 CSF specimens from patients thought to have tuberculous meningitis (TBM) were tested by the same PCR technique for tuberculosis and PCR results showed much more positivity than did microscopy or culture.

For the first time, ancient DNA of *M. leprae* was extracted and amplified from a bone dating from 600 AD.

The results illustrate the importance of tuberculin testing as a marker of *M. tuberculosis* infection and the value of leprosin A as a marker for *M. leprae* in long-treated leprosy patients. PCR results from *M. leprae* demonstrated the efficacy of immunotherapy for leprosy. The ELISA studies could differentiate between leprosy patients positive or negative by PCR for *M. leprae*. A ratio of antibodies to antigens of tuberculosis and leprosy, and G_0 were found to be valuable serological markers for tuberculosis in long-treated leprosy patients, and antibody to 65 kDa heat shock protein (hsp) was negatively associated with past immunotherapy with *M. vaccae*.

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PART TWO: DEVELOPMENT OF THE STUDY

LIST OF ABBREVIATIONS

AFB = acid-fast bacilli
AFM = acid-fast microscopy
BB = mid-borderline leprosy
BCG = bacille Calmette-Guerin
BI = bacterial index
BL = borderline lepromatous leprosy
bp = base pair
BT = borderline tuberculoid leprosy
cDNA = complementary DNA
CMI = cell-mediated immunity
CSF = cerebrospinal fluid
dATP = deoxyadenosine triphosphate
dCTP = deoxycytidine triphosphate
dGTP = deoxyguanosine triphosphate
DNA = deoxyribonuleic acid
dNTP = deoxynucleotide triphosphate
DTH = delayed type hypersensitivity
dTTP = deoxythymidine triphosphate
EIA = enzyme immunoassay
ELISA = enzyme-linked immunosorbent assay
ENL = erythema nodosum leprosum
G_0 = agalactosyl IgG
GuSCN = guanidine thiocyanate
HIV = human immunodeficiency virus
HLA = human leukocyte antigen
hsp = heat shock protein
I = indeterminate leprosy
IFN-γ = gamma interferon

IL = interleukin
IS = insertion sequence
IT = immunotherapy
kDa = kilodalton
LA = leprosin A
Lepr = leprosy
Lepr-PCR = leprosy PCR (PCR for *M. leprae*)
LJ = Lowenstein-Jensen
LL = lepromatous leprosy
MB = multibacillary
MDT = multi-drug therapy
MI = morphological index
NA = nucleic acid
OD = optical density
OT = old tuberculin
PB = paucibacillary
PCR = polymerase chain reaction
PGL-I = phenolic glycolipid-I
PNB = p-nitrobenzoate
PPD = purified protein derivative
RIA = radio immunoassay
RNA = ribonucleic acid
S = scrofulin
sec. tb = secreted *M. tuberculosis*
son. fortu = sonicated *M. fortuitum*
son. lepr = sonicated *M. leprae*
son. tb = sonicated *M. tuberculosis*
son. vacc = sonicated *M. vaccae*
T = tuberculin
T2H = thiophene-2-carboxylic acid hydrazide

Taq = *Thermus aquaticus*

TB = tuberculosis

TBM = tuberculous meningitis

TB-PCR = tuberculosis PCR (PCR for *M. tuberculosis*)

Th = helper T-cell

TNF = tumour necrosis factor

TT = tuberculoid leprosy

TU = tuberculin unit

V = vaccin

WHO = World Health Organization

ZN = Ziehl-Neelsen

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Figure 12. 1st metatarsal of a leprosy patient, with a normal control on left.

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PUBLICATIONS AND SHORT COMMUNICATIONS

Publications and short communications arising from this thesis:

Rafi A, Spigelman M, Stanford J, Lemma E, Donoghue H and Zias J. *Mycobacterium leprae* DNA from ancient bone detected by PCR. Lancet. 1994; 343: 1360-1361.

Rafi A, Spigelman M, Stanford J, Lemma E, Donoghue H and Zias J. DNA of *Mycobacterium leprae* detected by PCR in ancient bone. International Journal of Osteoarchaeology. 1994; 4: 287-290.

Rafi Abdolnasser, Donoghue Helen D, and Stanford John L. Application of polymerase chain reaction for the detection of *Mycobacterium leprae* DNA in specimens from treated leprosy patients. International Journal of Leprosy. 1995; 63: 42-47.

Rafi Abdolnasser, Baban Babak. Evaluation of direct microscopy, culture, and polymerase chain reaction for the diagnosis of tuberculous meningitis. Medical Journal of the I. R. Iran. In Press.

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PART ONE:

A CONCISE MYCOBACTERIOLOGY

CHAPTER 1

General Introduction

1.1. The genus *Mycobacterium*

The generic name *Mycobacterium* (fungus-bacterium) was introduced by Lehmann and Neumann in 1896. At that time the genus contained only two species, *Mycobacterium tuberculosis* and *Mycobacterium leprae.*

The genus *Mycobacterium* consists of aerobic, nonspore-forming, nonmotile, slightly curved or straight bacilli, 0.2 to 0.6 by 1 to 10 μm in size. The bacilli occasionally form branched filaments, but these can be readily disrupted. The cell wall is rich in lipids, making the surface hydrophobic and the mycobacteria resistant to many disinfectants and common laboratory stains. After staining, the mycobacteria are also resistant to decolorization with acid solutions, and for this reason they are named acid-fast bacilli. In view of the fact that the mycobacterial cell wall is complex and this group of microorganisms is fastidious, most mycobacteria grow slowly, dividing every 12 to 24 hours in culture. Isolation of the rapidly growing mycobacteria (e.g., *M. fortuitum, M. chelonae*) requires incubation for 3 days or more, whilst the slowly growing mycobacteria (e.g., *M. tuberculosis, M. ulcerans*)

require a 3 to 8 week incubation to isolate. *M. leprae*, the causative organism of leprosy, cannot be cultured *in vitro*. According to the approved lists of bacterial names (Skerman *et al.*, 1980), the genus *Mycobacterium* contained 41 species at that time (Table 1) though it has since been expanded. It has been shown that *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* are very closely related by antigenic analysis (Stanford and Grange, 1974), DNA hybridization (Baess and Bentzon, 1978), and numerical taxonomy (Tsukamura, 1983) and these mycobacteria should be combined into a single species.

Mycobacterial diseases are described in the Bible (e.g., leprosy) and in writings from ancient Greece (e.g., wasting diseases resembling tuberculosis). It is difficult to remember that tuberculosis was a leading cause of death 250 years ago. Tuberculosis today, is a global problem reponsible for the deaths of 3 million people each year (Kochi, 1991). The problem of leprosy extends beyond the number of active cases, estimated to be 5.5 million worldwide (Noordeen *et al.*, 1992). Tuberculosis and leprosy are two of the most dreaded diseases in the history of humankind and both remain major public health priorities of many developing countries. A number of species that usually exist as environmental saprophytes are also causes of human infections and are now being seen with increasing frequency. The mycobacteria that are associated with human disease are listed in Table 2.

Table 1. Approved mycobacterial names (Skerman *et al.*, 1980).

M. africanum
M. asiaticum
M. aurum
M. avium (the avian tubercle bacillus)*
M. bovis
*M. chelonae***
M. chitae
M. duvalii
M. farcinogenes
M. flavescens
M. fortuitum (the frog tubercle bacillus)
M. gadium
M. gastri
M. gilvum
M. gordonae
M. haemophilum
M. intracellulare
M. kansasii
M. komossense
M. leprae (the leprosy bacillus)
M. lepraemurium (the rat leprosy bacillus)
M. malmoense
M. marinum (the fish tubercle bacillus)
M. microti (the vole tubercle bacillus)
M. neoaurum
M. nonchromogenicum
M. parafortuitum
M. paratuberculosis (Johne's bacillus)
M. phlei
M. scrofulaceum
M. senegalense
M. simiae
M. smegmatis
M. szulgai
M. terrae
M. thermoresistibile
M. triviale
M. tuberculosis (the tubercle bacillus)
M. ulcerans
M. vaccae
M. xenopi

*The common names of some species are in parentheses
**Also termed *M. chelonei*

Table 2. Important mycobacteria associated with human disease.*

Mycobacterium	Environmental contaminant	Reservoir
M. avium	Yes	Soil, birds, swine, cattle, water
M. bovis	No	Cattle, humans, other mammals
M. chelonae	Yes	Water, soil, animals, marine life
M. fortuitum	Yes	Soil, water, animals, marine life
M. intracellulare	Yes	Soil, swine
M. kansasii	Yes	Water, cattle, swine (rarely)
M. leprae	Unknown	Humans, armadillos
M. malmoense	Probably	Unknown
M. marinum	Yes	Fish, water
M. scrofulaceum	Probably	Soil, water, foodstuffs
M. simiae	No	Primates, possibly water
M. szulgai	No	Unknown
M. tuberculosis	No	Humans
M. ulcerans	Probably	Unknown
M. xenopi	Yes	Water

*Human disease has also been rarely associated with other mycobacterial species.

1.2. Physiology and structure of mycobacteria

It has been revealed by freeze-fracturing techniques that the mycobacterial cell wall has several distinct layers (Barksdale and Kim, 1977). The mycobacterial cell wall is the most complex in all of nature, with the structural foundation formed by the peptidoglycan skeleton with covalently linked arabinogalactan-mycolate molecules and overlayed with free lipids (e.g., waxes, mycosides, cord factor) and polypeptides (Murray, 1990).

Mycobacterial lipids have been extensively investigated and several excellent and detailed reviews have been published (Goren and Brennan, 1979; Minnikin, 1982). Lipids are located on the outer layers of the cell and account for approximately 60% of the dry weight of the cell wall and confer properties that enable the organism to resist adverse environmental conditions (Willett, 1992). The lipids include waxes, species-specific mycosides (complex glycolipids and peptidoglycolipids), and cord factor (6,6"-dimycolate of trehalose). Cord factor is associated with the parallel alignment of rows of bacilli ("cord" formation), a characteristic sometimes associated with virulent strains of *M. tuberculosis* (Goren and Brennan, 1979). Phenolic glycolipids have been isolated from *M. kansasii*, *M. bovis* and *M. marinum* and are termed mycosides A, B and G respectively (Grange,1988). Large amounts of phenolic glycolipids similar to

mycoside A have been isolated from *M. leprae* (Hunter and Brennan, 1981). One of these, phenolic glycolipid-I (PGL-I) is specific for *M. leprae* and a monoclonal antibody has been produced against it (Young *et al.*, 1984).

The peptide chains in the outer layer comprise 15% of the cell wall weight and are biologically important antigens, stimulating the patient's cellular immune response to infection. Extracted and partially purified preparations of these and secreted protein derivatives are used as a skin-testing reagent to measure exposure to *M. tuberculosis*. Similar preparations from other mycobacteria have been used as species-specific skin-testing reagents (Magnusson, 1981), though more specific reagents can be prepared from bacterial cell sonicates (Stanford *et al.*, 1989a).

The peptidoglycan skeleton, the inner layer of the cell wall, overlying the cell membrane, is relatively uniform in all mycobacterial species and forms the major component of the cell wall. As with other bacteria, intrapeptide bridges between the peptidoglycan chains bind the skeleton into a rigid structure. Attached to these chains by covalent bonds are mycolic acids linked to D-arabinose and D-galactose. The mycolic acids are the major lipids in the mycobacterial cell wall.

Mycobacterial antigens may be soluble (cytoplasmic) and insoluble (cell wall lipid-bound). These antigens are classified according to their chemical structure or by their distribution within the genus. The

mycobacterial antigens are widely applied for identification and typing of the mycobacteria. Soluble antigens can be separated into four major groups as follows:

Group i, consists of antigens which are common to all mycobacteria; many of these antigens are also found in the nocardiae and some are detectable in related genera such as *Corynebacterium* and *Listeria.*

Group ii, consists of antigens which are detectable in slow-growing mycobacteria.

Group iii, comprises antigens which are found in rapid-growing mycobacteria, some of which are also found in the genus *Nocardia.*

Group iv, consists of antigens which are limited to individual species.

An advantage of such a classification is that non-cultivable mycobacteria extracted from tissues such as *M. leprae* and *M. lepraemurium* can be studied (Stanford, 1983).

Growth properties and colonial morphology are used for the preliminary identification of mycobacteria. *M. tuberculosis* and closely related species are slow-growing bacteria, and their colonies are nonpigmented. Runyon (1959) classified the other mycobacteria into four groups based on their rate of growth and their ability to produce pigments in the presence or absence of light. The pigmented mycobacteria produce intense yellow pigmented carotenoids. Photochromogens are organisms that produce these pigments only after exposure to light, whereas the scotochromogenic organisms can

produce the pigments in the dark as well as the light. The Runyon classification offers a useful guide to the most common mycobacterial isolates, although definitive identification requires biochemical or other types of testing. For laboratories with a simple mycobacterium culture facility, Runyon's system continues to be very useful, though it has long dropped from systems used by reference laboratories.

1.3. Pathogenicity and virulence of mycobacteria

The ability of a microorganism to cause disease is defined as its pathogenicity. Pathogenicity depends on the aggressiveness or virulence of the invading organism and the susceptibility of the host. In the case of mycobacteria, *M. tuberculosis* and *M. leprae* are obligate pathogens attacking man, having developed a total dependence on a living host for their continued existence. Many other mycobacteria are opportunist pathogens, normally existing as saprophytes but becoming pathogens under certain permissive conditions.

A quantitative measure of pathogenicity is defined as virulence. Virulence may vary considerably according to the host species. It has been recognized that mycobacteria owe their pathogenicity to their ability to invade and survive within the macrophages. Although some strains certainly liberate toxic compounds, their virulence is not

primarily associated with such substances (Grange, 1988).

It has been suggested that virulence of *M. tuberculosis* is related to the presence of certain toxic lipids in the cell wall. These include cord factor (Bloch,1950) and the sulpholipids (Middlebrook *et al.*, 1959). The nature of virulence of *M. leprae* is even less well understood. This organism is remarkably non-toxic. The virulence of *M. leprae* may well be attributable to its ability to enter non-immunological cells and to escape into the cytoplasm of macrophages. A further property of *M. leprae* that may be associated with its virulence is its ability to suppress DNA synthesis within an infected cell (Samuel *et al.*, 1987).

M. kansasii strains isolated from cases of human disease tend to be more resistant to hydrogen peroxide than strains isolated from the environment, suggesting an association similar to that postulated for *M. tuberculosis. Mycobacterium ulcerans* may owe its virulence to a cytotoxic substance, while virulence of *M. avium* appears to be related to colony mophology, suggesting a protective role for suface mycosides (Kuze and Uchihira, 1984).

1.4. The pathogenesis of tuberculosis

The rout of infection in tuberculosis is inhalation of infectious aerosols, each measuring about 2-10 μm in diameter and containing one or two

viable bacilli, which are able to reach the terminal airways. Following engulfment by alveolar macrophages, and providing there is no pre-existing immunity, the bacilli are able to replicate freely, with eventual destruction of the phagocytic cells. This leads to further cycles of phagocytosis by macrophages and lymphocytes that migrate to the site of infection, followed by cellular destruction. Infected macrophages also spread during the initial phase of disease to the local lymph nodes, as well as into the bloodstream and other tissues (e.g., bone, bone marrow, kidneys, spleen, central nervous system).

This process of slow growth, tissue destruction, and dissemination continues until the patient develops a cellular immune reaction to the organisms. This is heralded by the development of positive skin test reactivity to mycobacterial antigens. Although violent growth and tissue destruction can continue in progressive active tuberculosis, in most patients the cellular immune response arrests the the disease at this stage, with the only signs of infection a lifelong positive skin test and radiographic evidence of calcification of the initial active foci in the lungs or other organs.

Histological response to infection and growth of mycobacteria is characterized by granulomatous inflammation. When the tissue is initially infected, macrophages and polymorphonuclear leukocytes accumulate. After several weeks, and before an effective immune reaction is initiated, the macrophages become predominant, some of

which form multinuclear giant cells or "Langhans cells". In extensive active disease, cheesy (caseous) necrosis and cavitation are seen. This tissue destruction is mediated by the patient's response to growth of the bacilli, and possible sensitization of tissues to tumour necrosis factor (TNF) by bacterial products; no mycobacterial toxins have been described.

Reactivation of passive bacilli can develop years later when the patient's immunological responsiveness becomes weaker, either due to therapy or to immunosuppressive disease or old age (Citron and Girling,1985; Murray, 1990).

1.5. The pathogenesis of leprosy

Leprosy can induce a wide spectrum of disease activity characterized by its variations in clinical, histopathological, and immunological findings. Five forms of leprosy have been classified on the basis of these properties (Ridley and Jopling, 1966) as follows:

1. Tuberculoid leprosy (TT)
2. Borderline tuberculoid leprosy (BT)
3. Mid-borderline leprosy (BB)
4. Borderline lepromatous leprosy (BL)
5. Lepromatous leprosy (LL)

In this spectrum, only polar forms of TT and LL are stable. The other types are unstable, especially BT, which can swing to BB or BL in the absence of treatment. It has been shown that exposure to some environmental mycobacteria may influence the type of leprosy developed by susceptible individuals (Lyons and Naafs, 1987). Most leprosy cases can be exactly classified using the Ridley-Jopling or other methods, but from the point of view of therapy it is simpler to discuss them in terms of paucibacillary (PB) and multibacillary (MB) disease. Using the World Health Organization's definition (WHO Expert Committee, 1988), paucibacillary refers to patients with a bacterial index (BI) of 0 on the Ridley scale at all skin smear sites, and multibacillary to patients with a BI of 1+ or more at any site. Thus paucibacillary would usually include indeterminate, tuberculoid, and some borderline-tuberculoid cases. Multibacillary can be applied to the others.

Mycobacterium leprae is an obligate intracellular parasite that multiplies within the mononuclear phagocytes, especially the histiocytes of the skin and Schwann cells of the nerves. This bacillus has an especially strong propensity for nerves (Willett, 1992).

Tuberculoid leprosy (TT). In this form, the bacilli invade the nerves and selectively colonize the Schwann cells, multiply within them and slowly destroy them. The dermal nerve twigs are destroyed, and

the larger nerves are swollen and destroyed by granulomas and surrounded by a zone of lymphocytes: occasionally, there may be caseation within a dermal nerve (Ridley, 1974). The nerve damage is nonspecific and arises as a consequence of the cell-mediated immune response.

In tuberculoid leprosy, skin biopsy specimens show mature granuloma formation in the dermis that consists of epithelioid cells, giant cells, and rather extensive infiltration of lymphocytes. The tuberculoid granuloma leads to the nerve's destruction, and this in turn results in anaesthesia and/or muscle weakness, depending on the type of nerve involved. Acid-fast bacilli (AFB) are rarely seen (Willett, 1992; Jopling and McDougall, 1988).

Borderline types of leprosy (BT, BB, and BL). In these types, nerves are attacked in the same way as described for TT but higher concentrations of bacilli are required to elicit a cellular response, depending on the position of the patient in the borderline spectrum. The cellular response is less focal and less destructive, and microscopic examination reveals zones of epithelioid cells adjacent to areas of bacillated Schwann cells. Bacilli will be found within affected nerves, in small numbers at the tuberculoid end of the spectrum and in large numbers at the lepromatous end.

Histological examination of a skin lesion in borderline leprosy shows

characteristic features. In borderline-tuberculoid leprosy (BT) there is an epithelioid cell granuloma more diffuse than in TT with a free, but narrow, papillary zone. Giant cells tend to be of foreign body type rather than of Langhans type, and dermal nerves are moderately swollen by cellular infiltrate or may show only Schwann cell proliferation. AFB are usually absent from the dermis, but a few are likely to be found within dermal nerves. In mid-borderline leprosy (BB) there is a diffuse epithelioid cell granuloma with scanty lymphocytes and no giant cells. The papillary zone is clear and dermal nerves show slight swelling and cellular infiltrate. AFB are present within the dermis and within dermal nerves in moderate numbers. In borderline-lepromatous leprosy (BL) there is a macrophage granuloma in which some of the cells may show slight foamy change, and lymphocytes are present in dense clumps or are widely distributed in parts of the granuloma; a few epithelioid cells occasionally may be seen. Dermal nerves contain some cellular infiltrate. The papillary zone is clear, AFB are plentiful, distributed singly or in clumps, and sometimes in small globi (Jopling and McDougall, 1988).

Lepromatous leprosy (LL). The histopathology of lepromatous leprosy is notably different. Epithelioid and giant cells are absent, and lymphocytes are rare and diffusely distributed. The inflammatory infiltrate consists largely of histiocytes with a unique foamy appearance

resulting from the accumulation of bacterial lipids. Large numbers of AFB are found within the macrophages. It has been calculated that the number of bacilli within 1 cm^3 of infiltrated skin, in a lepromatous subject, varies between $1X10^9$ and $7X10^9$; the mean for 6 patients was $2.5X10^9$ (Hanks, 1945). Knowing the long generation time of *M. leprae* (possibly 10-12 days) it seems incredible that there can be such a build-up of bacilli in the space of a few years. *M. leprae* tends to invade vascular channels, which results in a continuous bacteraemia in lepromatous patients and consistent involvement of the reticuloendothelial system. The nerves are also infected, and numerous bacilli can be seen within the Schwann cells. Damage to the nerve structure, however, is less than in tuberculoid leprosy. Tissues bearing the brunt of the disease are: nerves; skin; eyes; reticuloendothelial system (this system includes lymph nodes and specialised cells of the liver, spleen, and bone marrow); mucosa of the mouth, nose, pharynx, larynx, and trachea; endothelium of small blood vessels; involuntary (plain) muscle, such as errector pili and dartos; skeletal (voluntary) muscle; and, in the male, testes. LL is considered to be the most infectious type of leprosy (Jopling and McDougall, 1988; Willett, 1992).

Indeterminate leprosy (I). This type is an early and transitory stage of leprosy. It is found in persons (usually children) whose

immunological status has yet to be determined. A scattered non-specific histiocytic and lymphocytic infiltration is seen that is diagnosable as leprosy in those cases in which there is a cellular reaction within a dermal nerve, or one or more leprosy bacilli are found in a situation, such as a dermal nerve, the subepidermal zone, or arrectores pilorum muscles (Jopling and McDougall, 1988). Subsequently, if not treated, indeterminate leprosy (I) can self heal, or progress towards PB or MB disease.

The importance of classification in leprosy. The reasons are as follows:

I. A correct classification will give essential information on whether a patient is infectious or not, on the prognosis, on the choice of chemotherapy and the length of treatment, and on the likelihood of complications, such as lepra reaction.

II. A classification which is widely known and applied enables clinicians to communicate intelligently with others.

III. When patients are selected for research projects, it is essential that their leprosy is accurately classified if faulty conclusions are to be avoided (Jopling and McDougall, 1988).

1.6. Immunity to tuberculosis

Infection of humans or susceptible animal species with virulent *M. tuberculosis* may lead to a range of manifestations from a self-limiting course with no clinical symptoms to full-blown tuberculosis with extensive granuloma formation at the site of infection. In both situations the infected individual will mount a delayed type hypersensitivity (DTH) reaction if tuberculin Purified Protein Derivative (PPD) is injected intradermally. In some cases the host is unable to neutralize the infection and the bacilli disseminate freely in the organism. This is the situation in miliary tuberculosis and in many Human Immunodeficiency Virus (HIV) positive individuals infected with *M. tuberculosis*. In these patients PPD may not evoke a DTH reaction (Andersen, 1994).

Protective immunity against tuberculosis is mediated primarily by the cellular branch of the immune system (Lurie and Zappasodi, 1942; Mackaness, 1968). It means that specific immunity to tuberculosis is due to presentation of mycobacterial antigen to helper T-cells, proliferation of helper T-cells, the subsequent production of macrophage-activating lymphokines, and the destruction of mycobacteria by the activated macrophages. It is therefore not surprising that AIDS patients, in whom HIV selectively has eliminated the CD4 positive T-cell population, are extremely susceptible to

tuberculosis, and that they often are unable to mount a DTH response (Barnes *et al.*, 1991). The DTH reaction is considered to be an indicator of the level of T-cell alertness of the individual.

The course of a mycobacterial infection is determined by the dynamic balance between host factors and properties of the infecting organisms and may lead to any degree of severity within a wide spectrum of possible manifestations. *M. tuberculosis* most often infects via the respiratory route. In the lungs the bacilli are phagocytosed by alveolar macrophages. One essential feature of virulent mycobacteria is that they, in some individuals, are capable of surviving and multiplying within the hostile environment of a macrophage (Andersen, 1994).

The concept of protective cell-mediated immunity (CMI) was established mainly by studies of mice infected with *Listeria monocytogenes*, but its application to tuberculosis raises several problems. The first problem is the relationship between CMI and the necrotizing reaction termed the Koch phenomenon, and a further problem is the exact role of the activated macrophage in the destruction of the tubercle bacillus. It has been difficult to prove experimentally that the activated macrophage can kill tubercle bacilli, however it is generally assumed (Grange, 1990). Human macrophages infected with *M. tuberculosis* demonstrate only a limited degree of inhibition (Steele *et al.*, 1986). It has been shown that gamma interferon (IFN-γ), one of the principal macrophage-activating lymphokines, may actually

increase the growth rate of *M. tuberculosis* in cultures of human macrophages (Rook *et al.*, 1986); but it should be borne in mind that macrophages may behave differently *in vivo.*

Intracellular reactions between the tubercle bacillus and the macrophage may lead to a state of bacterial dormancy or persistence. This phenomenon is virtually impossible to study *in vivo*, and dormant bacilli have probably never been seen (Stanford, 1987). Possibly, macrophages limit mycobacterial replication, and the subsequent lack of oxygen and nutrients, together with a low pH within macrophages and free fatty acids in the necrotic centre of the granuloma, lead to the eventual death of most bacilli.

In the absence of complications such as miliary or meningeal tuberculosis, the majority of primary complexes in man resolve. Progressive primary disease with the formation of a tuberculoma or a cavity is relatively uncommon. Macrophages destroy bacteria, or inhibit their growth, and secrete a wide range of immunologically relevant proteins, some of which are concerned in the pathogenesis of tuberculosis. Activated macrophages secrete proteases that liquefy caseous material within post-primary pulmonary lesions and thereby play a role in cavity formation. Activated macrophages also secrete TNF which may be responsible for the extensive necrosis seen in tissues sensitized by bacterial products in post-primary disease (Grange, 1990). TNF causes weight loss (Beutler and Cerami, 1986), and is

pyrogenic. TNF might therefore be the cause of fever and wasting characteristic of advanced tuberculosis.

The humoral immune response is not believed to be of importance in combatting mycobacterial infections (Lurie and Zappasodi, 1942) although this view is almost certainly naive. T-cells are clearly key elements in tuberculosis immunity. However, activated B-cells share with macrophages the ability to process and present antigen to specifically primed T-cells (Grey *et al.*, 1982).

1.7. Immunity to leprosy

Leprosy bacilli are less likely to give rise to clinical disease than are tubercle bacilli, yet skin-testing shows them to be highly infectious. Most individuals never develop clinical manifestations of disease, whereas many others develop a localized lesion that heals spontaneously. This implies that those individuals who develop progressive disease have innate or acquired immunological defects in their immunity to the leprosy bacillus (Willett, 1992). The T-cell mediated immune response plays a key role in leprosy. This is evident from the leprosy spectrum (Ottenhoff, 1994) which can be divided into groups far more clearly than in tuberculosis.

Tuberculoid leprosy (TT) patients mount a strong cellular immune

response (resulting in granuloma formation), and the number of bacilli observed in the lesions is low. TT patients show a strong delayed-type hypersensitivity to lepromin, and the histology of lesions is that of hypersensitivity granulomas (Andersen, 1994).

Lepromatous leprosy (LL) patients are loaded with bacilli and the T-cells are unresponsive to antigens from *M. leprae*. Borderline patients progressing to LL show a progressive loss of CMI and the development of an anergic state. Conversely, a high serological response characterizes LL. The high level of immunoglobulin in LL patients is by no means exclusively directed against the leprosy bacillus. There are numerous autoantibodies and antibodies directed against a whole range of substances apparently unrelated to the disease. This may well be due to *in vivo* polyclonal activation, and polyclonal hypergammaglobulinaemia can be a characteristic feature in LL. A number of abnormal serological activities are also associated with LL (e.g., false-positive VDRL test) (Bullock, 1978). Antibodies to *M. leprae* that cross-react with other mycobacteria may be detected in the sera of 75%-95% of the patients with LL. The part these humoral antibodies play in immune defence is unknown. Sometimes, erythema nodosum leprosum (ENL) occurs in MB patients, either spontaneously or triggered by treatment. It is associated with immune-complex deposition in the tissues, but it is unlikely that this is the initiating cause (Naafs, 1989).

Studies of CD4+ T-cells isolated from TT patients have disclosed that these cells preferentially produce mRNA encoding for IFN-γ, lymphotoxin, and IL-2, whereas lymphocytes isolated from LL patients appear to synthesize IL-4, IL-5, and IL-10 (Yamamura *et al.*, 1991). These findings show that the human T-helper lymphocyte (helper T-cell) population, has analogy with the murine system (Romagnani, 1991). It may be divided into at least two subpopulations: Th-1 and Th-2. In the murine system Th-1 clones mediate DTH reactions and the IFN-γ produced by them downregulates the Th-2 cells (Mosmann *et al.*, 1986; Cher and Mosmann, 1987). The function of Th-2 cells seems to be to provide help for B-cells and mutually to downregulate Th-1 cells explaining the high antibody levels found in LL. The factors that trigger the differentiation and activation of these T-cell subsets are as yet uncertain, but probably involve the the balance of relative concentrations of certain adrenal cortical hormones (Andersen, 1994; Daynes et al., 1991).

1.8. Immunotherapy for tuberculosis and leprosy

The aim of immunotherapy is to return the immune state to normal as soon as the majority of bacilli have been killed by chemotherapy and the circulating load of mycobacterial antigens has substantially fallen

(Stanford, 1989b). Immune mechanisms should then recognise and destroy persister bacilli, thus markedly shortening the period of chemotherapy required to prevent relapses and later reactivation.

A number of attempts have been made to treat tuberculosis and leprosy by stimulating the patient's immune reactivity. The first such attempt that appeared to be of value in the case of skin tuberculosis, was Koch's use of Old Tuberculin. The efficacy of vitamin D therapy in some cases probably also results from its role in macrophage activation (Grange, 1988).

Tuberculosis and leprosy patients fail to respond in skin-testing to the common mycobacterial antigens, suggesting that their responsiveness to the inducers of protective immunity has been switched off (Stanford *et al.*, 1981; Kardjito *et al.*, 1986). Immunotherapy with *Mycobacterium vaccae* restores the immune recognition of these common antigens and also regulates the tissue-necrotizing immune reactions (Nye *et al.*, 1986), suggesting that it might induce protective immunity and suppress antagonistic responses.

At present, the mechanism of *M. vaccae* immunotherapy is unknown, but it seems probable that it regulates the maturation of the various types of helper T-cells (Stanford and Grange, 1994). One possibility is that it suppresses Th-2 mediated responses and enhances Th-1 mediated responses (Stanford *et al.*, 1993) by blocking the influence of tumour necrosis factor on the pituitary/suprarenal axis

controlling circulating levels of dehydroepiandrosterone (Daynes *et al.*, 1991).

A number of studies so far strongly indicate that *M. vaccae* immunotherapy has great potential for the prevention and treatment of drug sensitive or drug resistant tuberculosis (Pozniak *et al.*, 1991). Results of *M. vaccae* immunotherapy investigations on leprosy patients in different countries have been more or less successful, but much needs to be done, and much remains to be found out (Stanford, 1994).

Reasons for the selection of *Mycobacterium vaccae* as an immunotherapeutic agent. *Mycobacterium vaccae* has never been associated with human disease, and claims for its pathogenicity for cattle are highly suspect. This organism is a dweller in moist soil and fresh water; it is rich in common, group i, mycobacterial antigens amongst which are thought to lie those that elicit protective immune responses. It lacks antigens of groups ii and iii (Stanford and Grange, 1974), and possesses its own species specific, group iv, antigens which are not known to have pathogenic significance. *M. vaccae* also possesses substances that may turn off the tissue necrotic elements of the Koch phenomenon (Nye *et al.*, 1986). This combination of immuno-modulating activity with the protective antigens lead to the selection of *M. vaccae* as an immunotherapeutic agent.

Immunotherapy with killed *M. vaccae* is a simple and potentially

very cheap adjunct to chemotherapy that can revolutionise the treatment of tuberculosis and leprosy (Stanford *et al.*, 1994; Stanford, 1994).

1.9. The epidemiology of tuberculosis

As mentioned earlier, tuberculosis is a global problem and it remains the leading cause of death (about 3 million deaths each year) among notifiable infectious diseases. In spite of the fact that effective methods for its control are available and have been applied successfully for several decades, in some regions the prevalence of tuberculosis is still inordinately high, and a plateau in the reduction of tuberculosis incidence is evident. It is estimated that around 50 million people have, or recently have had tuberculosis (Pallen, 1984). Most infectious cases are not diagnosed until after symptoms have developed. The premature collapse of clinical interest and awareness in low-prevalence areas has led to serious delays in diagnosis (Lundgren *et al.*, 1987). Although disease can be established in primates and laboratory animals such as guinea pigs, humans are the only natural reservoir. The tuberculosis case rates are influenced by the race, sex, and age of the population group. Primary exposure is generally restricted to selected populations such as the urban poor, patients with compromised immune systems

(e.g., AIDS patients), and immigrants from areas with a high incidence of disease. Medical care workers are also at risk for infection, because many patients with tuberculosis are admitted to hospitals without the disease being initially suspected. It seems that tuberculosis is the most common cause of death among institutionalised leprosy patients (Glaziou *et al.*, 1993).

In tuberculosis the route of transmission is close person-to-person contact through infectious aerosols. It has been shown that patients with sputum that is positive on direct smear examination are the main sources of infection (Rouillon *et al.*, 1976). Smear-negative patients, whether culture-positive or not, are of very low infectivity. The risk depends greatly on the closeness of contact as well as the infectiousness of the source case. On rare occasions, tuberculosis can also be acquired by ingestion or skin trauma. The most important source in transmission of tuberculous infection is the undiagnosed infectious person with cavitary tuberculosis. Patients receiving effective chemotherapy rapidly lose their infectiousness for other individuals (Grange, 1990; Willett, 1992).

1.10. The epidemiology of leprosy

Leprosy is considered important mainly because of its potential to cause

permanent and progressive physical deformities with serious social and economic consequences.

Estimation of the number of leprosy cases in the world is difficult. Case diagnosis and definition are not always clear or consistent and the enumeration of cases in many regions of the world is incomplete or irregular. Despite these difficulties, estimates are extrapolated from data from time to time. It was estimated that 12 million people suffer from active leprosy or its resultant disability (Pallen, 1984), but current progress suggests that this may have fallen by threequarters. Approximately $1.3X10^9$ people live in areas where leprosy is an important problem, i.e. where the estimated prevalence is over 1 case per 1000 persons, and thus may be considered at significant risk of contracting the disease (WHO, 1988).

Leprosy is known to occur at all ages ranging from early infancy to very old age. In endemic areas infection seems to occur mainly in children and young adults but, owing to the long incubation period of leprosy, diagnosis is often delayed till adulthood. Studies of sex distribution of leprosy show that although leprosy affects both sexes, in most part of the world males are affected more frequently than females, often in the ratio of 2:1 (Doull *et al.*, 1942).

Ethnic factors have been clearly demonstrated in different ethnic groups living in the same country or area. Such ethnic differences have been observed not only with regard to leprosy as a whole, but also with

regard to different types of leprosy, particularly in relation to the proportion of lepromatous leprosy. Ethnic differences have also been observed with regard to age and sex distribution. In Brazil, the disease was found to occur in more serious forms among white immigrants as compared with native Brazilians. In South Africa, leprosy was reported to occur in more severe forms among Europeans as compared with native Africans. In Malaysia, lepromatous leprosy is more common in Chinese than Malayans, Indians, and Europeans (Noordeen, 1994).

The occurrence of leprosy (and tuberculosis) more frequently in certain clusters, particularly family clusters, is well recognized. However, the most debated point is whether this is due to the clusters sharing the same environment or the same genetic predisposition, or a combination of both. The occurrence of leprosy in clusters has been particularly observed in low endemic areas.

It has been suggested that there is an association between leprosy and certain other factors such as climatic conditions, diet, nutrition, literacy and caste. The association between leprosy and hot and humid climates has been pointed out by some workers though the frequency of leprosy in Northern Europe and Asia in the last century belies this. Nutritional deficiencies and their relationship to leprosy have also been studied, notably Hutchinson association of leprosy with fish-eating (Hutchinson, 1906). However, none of the studies on the above factors have clearly established a causal association between them and leprosy.

However, in common with other mycobacterial diseases, protection from, and susceptibility to leprosy is significantly determined by prior contact with environmental mycobacteria. It is well recognized that socio-economic factors play an important role in leprosy. One of the striking features of the decline of leprosy in many parts of the world is its association with improved socio-economic conditions (Noordeen, 1994).

It has been shown that although leprosy is generally an endemic disease, occasionally it is capable of reaching epidemic proportions when conditions are favorable (Wade and Ledowsky, 1952).

Migration of leprosy patients may artificially reduce or increase the prevalence in some areas. Rural to urban migrations in some countries are not uncommon as a result of the social ostracism to which patients are exposed in their rural environment, and the attraction of economic opportunities in urban areas. Migration also facilitates introduction of new disease into unaffected areas.

Mortality in leprosy is often not considered important since the disease is rarely an immediate cause of death. However, leprosy patients are exposed to increased mortality risks due to its indirect effects. It has been shown that the mortality rate for LL patients is four times more than the general population, and that the situation for non-LL patients is very similar to that of the general population (Guinto *et al.*, 1954).

The human being appears to be the only reservoir of infection in leprosy, except for the fact that naturally occurring disease with organisms indistinguishable from *M. leprae* has also been detected among wild armadillos in parts of the southern United States (Walsh *et al.*, 1981). In Louisiana, up to 5% of armadillos have been found to have clinical disease, with about 20% having serological evidence of *M. leprae* infection (Truman *et al.*, 1986). Among human beings it is the LL cases that carry the largest load of organisms, as mentioned earlier, the maximum load reaching over $7X10^9$ organisms per gram of tissue. Patients with non-LL carry a very much smaller bacillary load, probably not exceeding 1 million organisms in total. In addition to clinically identified cases, the occurrence of AFB in the skin and nasal mucosa of healthy subjects have also been reported. The evidence that the AFB found on such carriers, is *M. leprae* is not conclusive, although there is some evidence that persons who carry such AFB, have a higher chance of developing the disease (Chatterjee, 1976; Chacko *et al.*, 1979).

The skin and the nasal mucosa are the main portals of exit of *M. leprae*. However, the relative importance of these two portals is not clear. It is true that LL cases show large numbers of organisms deep down in the dermis. However, whether they reach the skin surface in sufficient numbers is doubtful (Pedley, 1970). There is no doubt that when LL patients have ulcers from the breaking down of nodules or

when they have other breaks in their skin, large numbers of organisms could be discharged. It is also possible that small numbers of organisms escape to the surface of the skin along with sweat and sebaceous secretions. It has been reported that nasal secretions from LL patients can yield as many as 10 million viable leprosy bacilli per day (Davey and Rees, 1974).

The portal of entry of *M. leprae* into the human body is not definitely known. However, the skin and the upper respiratory tract are believed to be as the main portals of entry. It has been suggested that the leprosy bacilli would enter the system only through inoculation into the skin and not by passive skin to skin contact of inunction (Weddell *et al.*, 1963). Rees and McDougall (1977) succeeded in the experimental transmission of leprosy through aerosols containing *M. leprae* in immune-suppressed mice, suggesting a similar possibility in humans. Successful results have also been reported in nude mice when *M. leprae* were introduced into the nasal cavity through topical application (Chehl *et al.*, 1985). Such a possibility under natural conditions among human beings, has been questioned (Leiker, 1977). In summary, although no firm conclusions can be reached with regard to the portal of entry of leprosy, entry through the respiratory route appears most probable, although other routes, particularly broken skin, cannot be ruled out.

In leprosy, the exact mechanism of transmission in not known. Until

recently, it was believed that the disease was transmitted by contact between leprosy cases and healthy persons. More recently, the possibilities of transmission by the respiratory route, through ingestion, or by insects are gaining ground.

Regarding transmission by contact, two factors appear to be the most important, closeness of the contact and type of index case. In general, closeness of contact is related to the dose of infection, which in turn is related to the occurrence of disease. Of the various situations that promote close contact, contact within the household is the one that is easily identified. An interesting observation with regard to risk for contacts, which is relevant to closeness of contact, is the exceptional situation in Europe where immigrant cases and Europeans, who had returned home after acquiring leprosy in endemic countries, have failed to produce secondary cases among their contacts. With regard to contact and type of index case, it is well recognized that contacts of LL run a higher risk of getting the disease than contacts of non-LL, whose risk in turn is higher than that of non-contacts. With regard to duration of contact, in general, the longer the exposure of contacts to index cases the greater is the chance of getting the disease. While direct contact is expected to be more effective than indirect contact, the possibility of indirect contact playing an important role in the transmission of leprosy cannot be ruled out. This is particularly because of the possibility of the survival of *M. leprae* outside the human host, and the

possibility of contamination of clothing and other fomites by nasal secretions (Davey and Rees, 1974; Desikan, 1977).

The possibility of leprosy transmission by inhalation is based on (1) the inability of the organisms to be found on the surface of the skin, (2) the demonstration of a large number of organisms in the nasal discharge, (3) the high proportion of morphologically intact bacilli in the nasal secretions, and (4) the evidence that *M. leprae* could survive outside the human host for several hours or days. Successful transmission of infection to immune-suppressed mice through aerosols containing *M. leprae* indicates for transmission of leprosy through the respiratory route (Rees and McDougall, 1977).

Some experimental evidence has suggested the possibility of leprosy transmission through ingestion (Weddell and Palmer, 1963). The presence of *M. leprae* in the breast milk of mothers with lepromatous leprosy has also raised the possibility of infants acquiring infection through ingestion of breast milk. It has been calculated that a baby can receive as many as 2 million leprosy bacilli from a single feed from its lepromatous mother (Pedley, 1967). However, the epidemiological significance of this is not clear.

The question of whether insects actually transmit the infection has remained unanswered. However, it has been demonstrated that laboratory-bred Culex mosquitoes and bed bugs fed on lepromatous cases showed AFB in a high proportion, and that the AFB found were

M. leprae as verified through mouse footpad experiments. Leprosy bacilli have been found in pooled collections of insects found in the dwellings of leprosy patients. Also, *M. leprae* infection has been transferred from the insects to the mouse footpad by making the insects bite the footpads of the mice (Narayanan *et al.*, 1977).

The minimum incubation period reported for leprosy is as short as a few weeks and this is based on the very occasional occurrence of leprosy among young infants (Montestruc and Berdonneau, 1954). The maximum incubation period reported for leprosy is as long as 30 years or more. The long incubation period is clearly more important in situations where leprosy is on the decline. It may be more appropriate to call the interval between infection and disease in such instances the latent period (Noordeen, 1994) and to draw parallels with reactivation of persisters in tuberculosis.

Factors determining clinical expression after *M. leprae* infection. There is similar evidence in both leprosy and tuberculosis that not all people who get infected get the disease. The factors that determine clinical expression after infection appear to be as important as the factors that determine infection after exposure. Some of these factors that determine clinical expression of disease, are as follows:

Genetic factors. For a long time, genetic factors have been under

consideration, largely due to the observation of clustering of leprosy around certain families, and the failure to understand why certain individuals develop LL while others develop non-LL. Admittedly, host factors are believed to play a key role in this regard. One of the responsible genetic factors is located in the HLA system. Studies have shown that the susceptibility to certain types of leprosy, or, in other words, to different types of immunopathology, certainly is linked to HLA and associated with several HLA alleles, particularly with HLA class II alleles. Classical examples are HLA-DQ1, which is universally associated with LL, and HLA-DR3, which in some populations is associated with TT (De Vries *et al.*, 1976; Fine *et al.*, 1979; Fine, 1981; Van Eden and De Vries, 1984; Ottenhoff and De Vries, 1987).

Route of infection. Studies in the mouse footpad model suggest that the route of entry of the leprosy bacillus may, to some extent, determine the occurrence of leprosy. This is based on the observation that while intradermal administration of killed *M. leprae* sensitizes the animal, intravenous administration of killed *M. leprae* tends to tolerize the animal, as studied through skin test reactivity. This also raises the possibility of TT and LL being the result of different routes of entry of the bacteria (Shepard *et al.*, 1982).

Re-infection. The occurrence of leprosy, presumably for the first

time, in older individuals in endemic areas has raised the possibility of re-infection in these individuals, since it is difficult to believe that they remained uninfected for such a long time in an endemic area. However, this occurrence in older age groups can also be explained by the possibility that the disease in these persons represents reactivation of old undetected primary disease following waning of previously acquired immunity. Since there is no evidence of a distinct primary disease occuring in leprosy as in tuberculosis, the hypothesis of re-infection gains some importance. Further, the occurrence of relapse in leprosy suggests, at least in a proportion of relapsed individuals, the possibility of re-infection. There is nothing to prevent these immunedeficient inactive patients living in endemic areas from succumbing to fresh infection. In the absence of a method for the identification of strain variations of *M. leprae*, the hypothesis on re-infection will remain untested (Noordeen, 1994).

Prior infection with other mycobacteria. Some studies indicate that as in tuberculosis, the environmental mycobacteria and possibly *M. tuberculosis* play a role in the occurrence of leprosy. This is possibly due to antigenic overlap between *M. leprae* and other mycobacteria. The varying degrees of protection given by BCG against leprosy in different geographic areas, and the limited protection seen among natural tuberculin positive reactors in the BCG study, support this

possibility (Stanley *et al.*, 1981). Further, it has been suggested that the protective efficacy of BCG in different areas may be enhanced or diminished, depending upon the local environmental mycobacteria, some acting synergistically with BCG and some antagonistically (Rook *et al.*, 1981).

HIV infection and leprosy. It has been shown that HIV infection has created a serious situation with regard to the incidence of tuberculosis. Case control studies done in several parts of Africa have clearly shown that the substantial increase in pulmonary tuberculosis is attributable to HIV infection (Orege *et al.*, 1993). The same situation has been recognized for atypical mycobacterioses. With regard to leprosy, there is no clear information so far, although a similar situation is possible. There is very little information on the interaction between HIV and leprosy, especially concerning the clinical progress of leprosy (Bwire and Kawuma, 1993; Blum *et al.*, 1993). It is not clear whether or not this is a result of coincidence. Only good case control studies can provide an answer to the question of HIV infection as a risk factor for clinical leprosy.

When a patient acquires both HIV and the leprosy bacterium, both infections may establish themselves. HIV infection slowly destroys the CMI and *M.leprae* multiplies unchecked. Since leprosy, with the exception of polar LL, needs a functional CMI in order to develop overt disease, it may well be that the clinical symptoms of leprosy in an

HIV-infected patient do not develop. Or, when they do develop, they may be inconspicuous as demonstrated by the reported patient. An HIV-infected leprosy patient should be considered as a downgrading leprosy patient. Further investigations have to continue to elucidate the relationship between HIV and *M. leprae* infection. Therefore, diagnostic methods for the detection of a leprosy infection such as serology and polymerase chain reaction (PCR) should be developed further (Naafs *et al.*, 1994).

CHAPTER 2

Methods for the Laboratory Diagnosis of Tuberculosis and Leprosy

The decade of the 1990s may well represent a bridge between the past and the future in the laboratory diagnosis of tuberculosis and leprosy, a bridge between the morphological approach of the past into the beginning of an era of nonculture direct detection using the polymerse chain reaction (PCR).

The applicable and the useful methods for the laboratory diagnosis of tuberculosis and leprosy can consist of three steps, as follows:

I. Diagnosis based on the conventional methods (microscopy and culture).

II. Diagnosis based on the response of the host to the aetiologic agent (skin test and antibody detection).

III. Diagnosis based on the molecular techniques (DNA probes and PCR).

2.1. Diagnosis based on the conventional methods (microscopy and culture)

Examination of clinical specimens from tuberculosis patients by

microscopy and culture, and also microscopic examination of clinical samples from leprosy patients, are routinely carried out (in clinical laboratories).

In tuberculosis, the collection of adequate specimens is essential for reliable bacteriology. Clinical specimens are collected in sterile wide-mouthed screw-capped 'Universal' containers. Sputum samples are collected on waking in the morning. The patient is instructed to cough vigorously after deep inspirations, repeatedly, if necessary, until at least 3 ml of sputum has been produced. If sputum cannot be produced, pharyngeal secretions mixed with saliva should be obtained, after clearing the back of the throat. In children, or patients who are unable to co-operate in providing sputum or pharyngeal secretions, gastric contents may be aspirated. Cerebrospinal fluid (CSF), pus, and other fluid specimens should be of as large a volume as possible for the laboratory to stand a good chance of isolating the bacilli (Citron and Girling, 1985).

In leprosy, the laboratory diagnosis may be suspected from the symptoms, from the type and distribution of the lesions, and from a history of having lived in an endemic area. Specimens used for the diagnosis of leprosy can be skin lesions, nasal scrapings, ear lobes, tissue secretions, nasal secretions, and sputa (Willett, 1992; Rees and Smith, 1990; Laidlaw, 1989), and the diagnosis may be made by the demonstration of acid-fast bacilli (AFB) in smears of such specimens.

The most readily available tissue from which to obtain *M. leprae*, by means of the slit-skin smear technique, is provided by the skin (Rees and Smith, 1990). Specimens may be obtained from any ulcerated nodule on the skin. An unulcerated nodule is slit with a scalpel and squeezed to exude fluid on to a microscope slide. A specimen obtained from a slit in the ear lobe may yield a positive result even when there is no obvious local lesion. When skin lesions are not apparent, specimens should always be obtained from scrapings or secretion from the nasal mucosa, for these may yield positive results. *M. leprae* may also be present in sputum when the lungs are affected (Laidlaw, 1989). Sputum samples collection should be the same as in tuberculosis, as mentioned above.

Specimen processing for the recovery of AFB from clinical specimens involves a number of complex procedures, each of which must be performed with precision. Specimens from sterile sites can be directly used for diagnosis (small volume) or concentrated to reduce volume. Other specimens such as sputum samples require decontamination and concentration (Baron *et al.*, 1994).

Microscopy. Detection of acid-fast bacilli in clinical specimens is extremely valuable for rapid laboratory confirmation of tuberculosis, leprosy, and other mycobacterial diseases. Sputum smears are prepared by selecting purulent particles of the specimen and smearing them

direct on to a microscope slide using a wire loop. Then sputum smears are dried, fixed, and stained (Murray, 1990; Citron and Girling, 1985). Some workers examine selected purulent or mucopurulent portions of sputum directly while others attempt to concentrate the bacilli by centrifugation after digestion of sputum with *N*-acetyl-L-cysteine or some other mucolytic agent (Grange, 1990). Microscopic examinations play an important role in tuberculosis control programmes because most patients with symptomatic tuberculosis will demonstrate AFB in the sputum (Willett, 1992), and on the other hand, microscopic examination is much cheaper and provides more valuable information than repeated chest radiography (Citron and Girling, 1985).

Two methods are currently used for microscopical examination of the clinical specimens, bright field microscopy after Ziehl-Neelsen (ZN) staining and fluorescence microscopy after staining with auramine O or auramine-rhodamine, both of which methods make use of the acid-fastness of mycobacteria. The acid-fast stain binds strongly only to bacteria that have a waxy material in their cell walls, therefore this stain is used to identify all bacteria in the genus *Mycobacterium*, including the two important disease producers *M. tuberculosis* and *M. leprae,* and also the disease-producing strains of the genus *Nocardia* (Citron and Girling, 1985; Tortora *et al.*, 1992).

M. leprae is not as strongly acid-fast as *M. tuberculosis*, hence to demonstrate the leprosy bacilli, the ZN staining method should be

modified by the use of a weaker decolourizer than used for tubercle bacilli; 5% H_2SO_4 should be used instead of 20% H_2SO_4, or 1% HCl in alcohol may be used (Laidlaw, 1989).

Slit-skin smears after ZN staining, are examined with a X100 magnification objective (eyepiece X6 or X8). The number of AFB per field is determined after examining 25-100 fields, on a logarithmic scale ranging from 0-6.

The number of bacilli are expressed as the bacterial index (BI) to measure the weight of infection, as follows:

BI	Number of AFB
6+	more than 1000 bacilli in each microscopic field
5+	100 to 1000 bacilli in each field
4+	10 to 100 bacilli in each field
3+	1 to 10 bacilli in each field
2+	1 to 10 bacilli in 10 fields
1+	1 to 10 bacilli in 100 fields
0+	0 bacilli in 100 fields

Serial smears will indicate the response of the patient to chemotherapy. Serial smears can also be used to assess the morphological index (MI), which gives a measure of the viability of *M. leprae* on the basis of morphology; uniformly staining bacilli are probably alive, whereas

irregularly staining bacilli are dead (Grange, 1988; Rees and Young, 1994; Rees and Smith, 1990). The leprosy bacilli are typically found tightly packed within macrophages, but some extracellular bacilli can also be seen.

With regard to tuberculosis, a report from the laboratory should provide an estimate of the number of AFB detected. The American Lung Association has recommended the following method (Willett, 1992):

Number of AFB	Report
0	No AFB seen
1-2/slide	Report number found and request repeat specimen
3-9/slide	Rare or +
10 or more/slide	Few or + +
1 or more/field	Numerous or + + + +

Culture. Except for *Mycobacterium leprae*, all named mycobacteria can be cultured *in vitro*. *M. leprae* has not been cultured outside the animal cell, and the reasons for this are not at present evident (Draper, 1983).

Isolation of mycobacteria from clinical specimens is complicated by the fact that most isolates grow slowly and can be obscured by the

rapidly growing bacteria normally present in clinical specimens. Thus specimens such as sputum are initially treated with a decontaminating agent (e.g., *N*-Acetyl-L-cysteine plus 2% NaOH, Dithiothreitol plus 2% NaOH, etc). Because mycobacteria are tolerant to brief alkali treatment, this process selectively kills the rapidly growing bacteria. Extended decontamination of the specimen will kill mycobacteria, so the procedure is not performed with normally sterile specimens or when small numbers of mycobacteria are expected (Murray, 1990; Koneman *et al.*, 1992).

For growth on culture, *M. tuberculosis* requires a nitrogen source such as glutamate or asparagine, either glucose or glycerol as a source of carbon, and essential metals such as iron and magnesium. Lowenstein-Jensen (LJ) medium is a whole-egg medium and, without potato starch, is probably the most widely used medium for culturing *M. tuberculosis* in standard bacteriological tests. Traditionally, specimens are inoculated onto LJ medium which is usually incubated for at least 8 weeks, with weekly inspection for growth. Most strains of *M. tuberculosis* appear within 4 weeks, but they may not be visible for 8 weeks or more if they originated from patients treated with antituberculous agents. The detection time has recently been shortened by the use of specially formulated broth cultures where the metabolism of ^{14}C-labelled palmitic acid is measured in an ion chamber system (BACTEC) (Citron and Girling, 1985; Grange, 1990; Murray, 1990).

A good culture service should be available in all countries because it can increase the proportion of bacteriologically confirmed tuberculosis by 30-35 per cent over those diagnosed by microscopy alone.

The cultural and metabolic properties of *M. tuberculosis* distinguish it from other mycobacteria. Thus, *M. tuberculosis* is characterized by colonial morphology,slow growth, failure to produce pigment, oxygen preference, absence of growth on LJ medium containing 500 mg of p-nitrobenzoate (PNB) per litre, and by absence of growth at 25°C. On LJ medium colonies of *M. tuberculosis* are large, heaped up and termed eugonic while those of *M. bovis* are small, flat and termed dysgonic although their growth is stimulated by pyruvate, making the colonies larger. Colonies of *M. tuberculosis* lie on the surface while those of *M. bovis*, because they can tolerate a lower oxygen tension, extend into the surface layers. *M. tuberculosis*, unlike *M. bovis,* produces nicotinamide (niacin), reduces nitrate to nitrite, is resistant to thiophene-2-carboxylic acid hydrazide (T2H), and is sensitive to pyrazinamide, unless resistance has been acquired.

Positive cultures should be screened to find out whether they are *M. tuberculosis* or *M. bovis*, or another mycobacterial species. Some recommended screening tests are shown in Table 3. The full identification of non-tuberculous mycobacteria involves specialized procedures (Citron and Girling, 1985).

Table 3. Screening tests for identifying cultures of mycobacteria (Citron and Girling, 1985).

Organism	Growth at 25 °C	Growth on PNB*	Niacin production
M. tuberculosis	-	-	+
M. bovis	-	-	-
Other mycobacteria	+	+	-

*PNB, p-nitrobenzoate

2.2. Diagnosis based on the response of the host to the aetiologic agent (skin test and antibody detection).

Diagnostic methods based on the response of the host to the aetiologic agent are used to detect tuberculosis and leprosy infection or disease. Although these methods lack specificity and sensitivity, these tests are epidemiologically useful in chronic and extensive disease.

Skin test. Reactivity to intradermal injection of mycobacterial antigens can differentiate between infected and noninfected individuals. Tests with antigens extracted from *M. tuberculosis* have been used most commonly and are best standardized, although skin tests with species-specific antigens from other mycobacteria have also been developed.

Stanford and his colleagues (1975) adopted a much more rational approach to the preparation of skin-testing reagents, resulting in the production of the 'New Tuberculins'. These reagents are prepared by harvesting mycobacteria during the growing phase from non-antigenic media, washing thoroughly, disrupting the cell mass in an ultrasonicator, sterilizing by repeated membrane filtration, and diluting to a suitable protein concentration.

Skin-testing in tuberculosis. The tuberculin skin test plays an important role in the control of tuberculosis. The test identifies the

majority of cases of tuberculosis infection, recent or past, with or without disease. The tuberculin skin test is based on the individuals infected with *M. tuberculosis* developing hypersensitivity to the proteins of the bacillus. Unfortunately a reaction to tuberculin does not always indicate infection by *M. tuberculosis*, since many of its antigens are common to other mycobacteria.

Two preparations of tuberculin antigens are currently used: old tuberculin (OT) and purified protein derivative (PPD). Standardized PPD is recommended and this is the skin test reagent that is primarily used to detect hypersensitivity to *M. tuberculosis* nowadays. Accurate standardization in man against the designated standard PPD-S has been arbitrarily designated as 50,000 tuberculin units (TU) per mg of protein (Willett, 1992).

Skin-testing in leprosy. In leprosy, skin-testing is useful epidemiologically as a guide to the extent of exposure of a community to the leprosy bacillus. It is also of value in determining the position of the patient on the immunologic spectrum and it may be a marker of protective immunity.

Two types of skin-testing reagents are used: lepromins (Mitsuda or integral lepromins) and leprosins. Lepromins are heat-killed (autoclaved) suspensions of *M. leprae* prepared from lepromatous nodules. Leprosins are sonicated and filtered soluble antigens derived

from leprosy bacilli separated from mammalian tissues. The suffix A or H indicates whether the reagent is derived from armadillo or human material respectively. Dharmendra type lepromin is a tissue-free bacillary extract of human origin and used on a wide scale, but many workers prefer to use the armadillo-derived equivalent.

After intradermal injection of the skin-testing reagents, two different reactions occur: the early (Fernandez) reaction and the late (Mitsuda) reaction. The early reaction (Fernandez reaction) is seen particularly with the leprosin type reagents. It resembles the tuberculin reaction, a delayed type hypersensitivity (DTH) and appears 24 to 48 hours after testing and is read after 48 to 72 hours as diameter of induration. Tissue necrosis does not occur. In common with the tuberculin reaction, a positive reaction indicates previous exposure to the relevant antigens, although many antigens in the reagent are shared by all mycobacterial species. Thus the leprosin test may be positive in healthy contacts and to a large extent in non-contacts as well as in immunologically reactive patients.

The late reaction (Mitsuda reaction) is seen with the lepromin type reagents. It appears 10 days or more after testing and is usually measured after 3 weeks. The Mitsuda reaction may not be a measure of pre-existing DTH, but is the manifestation of cell-mediated immunity (CMI), which the lepromin itself has induced. Lepromin reactivity is strongest in TT patients, weaker in BT patients but negative in BB, BL

and LL patients. Positive lepromin test results occur in patients with tuberculosis and 70% or more of healthy persons in areas of the world where no leprosy exists. Also, positive reactivity can be induced in normal, healthy children by vaccination with BCG. However, Mitsuda reaction is used throughout the world as a skin test reactivity for the classification of leprosy and assessment of immune responsiveness to *M. leprae* in leprosy patients, rather than as an epidemiological tool (Grange, 1988; Willett, 1992; Rees and Young, 1994).

Skin-testing can also be used to assess immunotherapy in a very simple way, but requires the investigation of quite large numbers of patients before it is valuable, and cannot be applied usefully until a year or more after the immunotherapy has been given (Stanford, 1994).

Antibody detection. Attempts have been made to develop reliable immunoassays for tuberculosis and leprosy based on the detection of antibody in human serum.

An immunoassay is a technique for measuring the presence of a substance using an immunological reaction. While this could be used to describe the proliferation of lymphocytes in culture, or the wheal and flare response of the skin of an allergic individual when allergen is injected, it is almost exclusively used to describe tests that exploit the reaction between antibody and antigen *in vitro*.

Immunoassays include simple precipitation of antibody/antigen

complexes, agglutination of coated red cells or other particles, electrophoretic immune precipitation in agar, radio immunoassay (RIA) and enzyme immunoassay (EIA). The development of the enzyme-linked immunosorbent assay (ELISA) is best appreciated in the context of the preceding RIAs. All ELISAs employ enzyme-labelled conjugates, which were first investigated by Avrameas and Uriel (1966), and Nakane and Pierce (1966) for use in the localization of antigens in tissue. These enzyme-labelled conjugates were found to be at least as sensitive as fluorescein-labelled antibodies for detecting cellular antigens (Avrameas, 1969). It was soon discovered that enzyme-labelled antigens and antibodies could be used to quantify various components of serum (Avrameas and Guilbert, 1971; Engvall and Perlmann, 1971; Van Weeman and Schuurs, 1971); in 1971 Engvall and Perlmann, and Van Weeman and Schuurs, working independently, were the first to report the development of ELISAs.

ELISAs have been shown to be at least as sensitive as most of the other techniques (Wreghitt *et al.*, 1984), and have particular advantages over tests such as RIA, notably the long shelf life of conjugates, no radioactivity hazards and less costly equipment. Although the ELISA can be performed as a competitive binding test such as RIA, most applications employ the indirect method for antibody detection and the double antibody sandwich method for detection of antigen (Engvall and Perlmann, 1971).

In indirect ELISA (indirect method for antibody detection), an antigen specific for the antibody to be measured is bound to a solid phase (the solid phase may be multiwelled plates, tubes, or polystyrene beads). After incubation the solid phase that is stable at 4°C is washed to remove the unbound antigen. Dilutions of the test serum are added, incubated, and then washed. An enzyme-labelled (usually peroxidase) anti-species antibody is added and incubated. Unbound conjugate is removed by washing. The enzyme substrate is added, and the reaction is terminated after the colour reaction has occurred.

In the double antibody sandwich method (for antigen detection), the antibody is bound to the solid phase. Excess antibody is removed, and the coated solid phase is stored at 4°C. At the time of the test, the coated solid phase is removed and incubated with the antigen-containing specimen. After incubation, unbound antigen is removed by washing. Specific antibody-enzyme conjugate is added and incubated , and the excess is removed. The enzyme substrate is added, and the colour reaction is read.

2.3. Diagnosis based on molecular techniques (DNA probes and PCR)

The use of nucleic acid hybridization analysis with DNA probes

specific for particular mycobacterial pathogens has become widespread in recent years because it offers rapid, specific diagnosis with a relatively high degree of sensitivity. However, more sensitive DNA detection techniques of mycobacteria especially *M. tuberculosis* and *M. leprae* employing the DNA amplification methodology referred to as the polymerase chain reaction (PCR) have recently been used.

DNA probes. Nucleic acid hybridization tools termed DNA probes have been developed for rapid identification of mycobacteria. For example, DNA extracted from *Mycobacterium* is broken into fragments by using a restriction enzyme, and a specific fragment is selected as the probe for *Mycobacterium.* This fragment must be able to hybridize with the DNA of all *Mycobacterium* strains but not with the DNA of closely related mycobacteria or other bacteria. The chosen DNA fragment is cloned in a plasmid in *Escherichia coli*, producing hundreds of specific *Mycobacterium* DNA fragments. These fragments are tagged with radioactive isotopes or a fluorescent dye and are separated into single strands of DNA. The resulting DNA probes can then be mixed with single-stranded DNA from a sample suspected of containing *Mycobacterium.* If *Mycobacterium* is present, the DNA probes will hybridize with the *Mycobacterium* DNA, and hybridization can be detected by the radioactivity or fluorescence of the probes.

At present, the use of probes is limited to detection of the organisms

following culture. In the detection of mycobacteria, the sensitivity of probes directly in clinical samples is approximately 100 times less than culture and false positive results can occur (McFadden *et al.*, 1990; Desmond, 1992).

The polymerase chain reaction (PCR). PCR is a method of amplifying small quantities of nucleic acid (DNA or RNA) to the point where they are readily visualized.

In 1985, Kary Mullis invented the PCR technique (Mullis, 1990). He thought of using two oligonucleotide sequences, oppositely oriented, and a DNA polymerase enzyme, to double the number of DNA targets. Each product would then become the target for the next reaction, effectively yielding a product which doubled in quantity with each repeated cycle. The invention of this amplification system quickly became known as the polymerase chain reaction (PCR). Specific DNA sequences from as few as 25 base pairs up to 10,000 base pairs in length from the entire genome can be amplified by PCR (Saiki *et al.*, 1988). In fact, this invention was one of the rare advances in the field of molecular biology, which is not only stunningly simple, but also of vital importance in the development of molecular biology. Originally, the PCR technique was applied by workers in the Human Genetics Department at Cetus to the amplification of human β-globin DNA and to the prenatal diagnosis of sickle-cell anaemia (Saiki *et al.*, 1985; Saiki

et al., 1986; Embury *et al.*, 1987).

Because of several reasons, the use of molecular biological techniques have still made little or no impact on the work of most clinical laboratories, in spite of considerable use of these techniques in the research laboratory. These reasons are: the poor sensitivity of such probes, particularly when compared to culture; the labour-intensive procedures involved in their use; and reluctance to use radioactivity in a routine setting. The advent of DNA amplification using PCR has improved these shortcomings.

Its exquisite in sensitivity, together with increasing ease of performance, has placed PCR in a central position in molecular biological research. PCR promises to achieve similar status in clinical diagnosis. Using PCR, scientists have been able to analyse DNA samples from individuals, even before birth; and from samples taken many thousands of years after death. Clinical geneticists can assess an individual's genetic status using a few buccal epithelial cells obtained from a mouthwash sample by performing PCR. PCR has meant that the forensic scientists can now identify a criminal from a single hair. PCR has enabled taxonomists to classify extinct or non-cultivable organisms, and virologists and bacteriologists to detect difficult pathogens (Rolfs *et al.*, 1992).

Nowadays, amplification of mycobacterial DNA by PCR has provided a new tool for rapid and accurate mycobacterial disease

diagnosis and epidemiological studies, particularly in tuberculosis and leprosy.

The principles of PCR. As is well-known, DNA is a double helix molecule with two strands, running in opposite directions, in an antiparallel arrangement. The two strands of DNA are specifically bound to one another by hydrogen bonds between complementary bases (C binding to G with three bonds and A binding to T with two bonds). The two strands of DNA molecule can be separated by heating to give two single-stranded DNA molecules, a process known as denaturation. Single-stranded molecules can bind to one another through the specific interactions between complementary bases, a process known as annealing or hybridization.

The above processes apply equally to DNA probes and PCR, but, an additional process that is required to complete PCR, is extension. If a short single-stranded DNA fragment (a primer) is annealed to a longer single-stranded template molecule, a thermostable DNA polymerase, such as that of *Thermus aquaticus* (Taq) will be able to bind to the 3' end of the primer and extend it to synthesize a new complementary DNA strand using deoxynucleotide triphosphates (dNTPs= dATP, dCTP, dGTP, and dTTP). This process is known as extension (Figure 1).

The short single-stranded DNA molecules (oligonucleotides) are

quickly and cheaply synthesized by chemical means. When the oligonucleotides are added to denatured DNA, they act as primers for DNA polymerase, and allow the new DNA to be synthesized *in vitro*.

Two oligonucleotide primers are used in the PCR assay, which hybridize to specific sites on opposite strands of the target DNA (Figure 2). When the DNA polymerase extends a primer, it makes a DNA which contains a new binding site for the other primer (Schochetman *et al.*, 1988; Persing, 1991).

The cycling process of PCR and its performance. As mentioned above, basically each cycle of PCR consists of three steps (Figure 2):

1. Denaturation. This step separates the complementary strands of double-stranded DNA (target DNA) by heating to high temperature. Initial heating of the PCR mixture at 94°C for 2 to 5 min is enough to completely denature complex genomic DNA. However, heat damage of DNA leads to an increased nucleotide misincorporation rate during PCR (Eckert and Kunkel, 1991).

2. Annealing. In this step, primers are attached to the separated DNA strands (target molecules) after cooling the reaction. Each primer is complementary to one of the original DNA strands, to either the 5' or 3' end of the sequence of interest. Annealing temperatures in the range of 55°C to 72°C for 30 sec to 2 min generally yield the best

results (Schochetman *et al.*, 1988; Innis *et al.*, 1990).

3. Extension. In this step, DNA polymerase (Taq polymerase) extends primers to synthesize new DNA using dNTPs. Primer extensions are performed at 72°C (optimum temperature for DNA synthesis). This temperature is suitable for the activity of the Taq polymerase. Extension time depends upon the length and concentration of the target sequence and upon temperature, and it can last from a few seconds to a few minutes (Innis *et al.*, 1990).

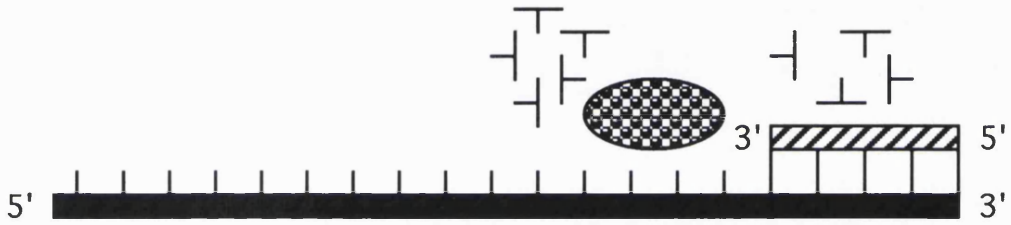
During each PCR cycle, there is a doubling of the number of copies of the target DNA sequence (Figure 2). Repeating the PCR cycle many times (20-50) causes the target DNA sequence to amplify a million fold or more. The optimum number of cycles will depend mainly upon the starting concentration of the target DNA when other parameters are optimized. Too many cycles can increase the amount and complexity of nonspecific background products, and too few cycles give low product yield.

Usually, after the last PCR cycle, a 5-15 min 72°C hold time is performed to promote completion of partial extension products and complete the annealing of single-stranded complementary products (Innis *et al.*, 1990; Rolfs *et al.*, 1992).

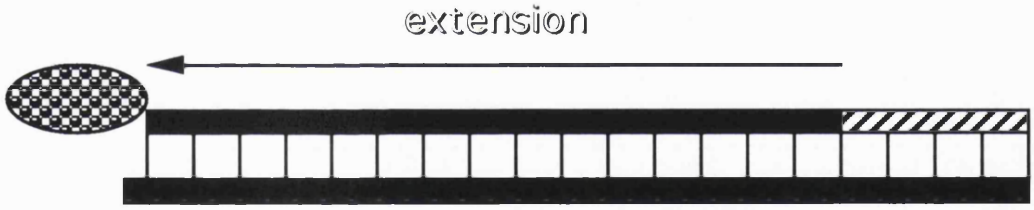
Since the invention of PCR, two technical advances have notably improved it. The first advance was the development of thermostable polymerases such as Taq polymerase, which have the capability of

resisting the denaturation temperature (Saiki *et al.*, 1988). This means that it is now possible to perform PCR without opening the tubes and adding fresh enzyme after each denaturation step. The second advance has been the commercial development of programmable heating blocks (thermal cyclers). This advance has eliminated the tedious and time consuming process of moving the PCR tubes from one water bath or heating block to the next one. These two advances enable us to mix all the PCR components (PCR buffer, two primers, four dNTPs, Taq polymerase, and template DNA or template cDNA) together in polypropylene tubes, and overlay the mixture with mineral oil (Figure 3), and then leave the tubes unattended in a thermal cycler for the few hours needed for amplification to take place. The presence of the amplified products is detected by electrophoresis of the amplified mixtures on an agarose (or a polyacrylamide) gel, staining the amplified products with ethidium bromide, and visualizing them on a UV transilluminator. Under such conditions, the nucleic acids are seen to be fluorescing, and can be recorded by photography. To determine the size of the amplified product, a known molecular weight marker is run with the PCR products in the same gel (Schochetman *et al.*, 1988; Maniatis *et al.*, 1983).

Single-stranded primer anneals to single-stranded DNA



DNA polymerase recognises 3' end of primer



DNA polymerase synthesizes new DNA strand from dNTPs, using complementary strand as template

Figure 1. Primer extension by a DNA polymerase.

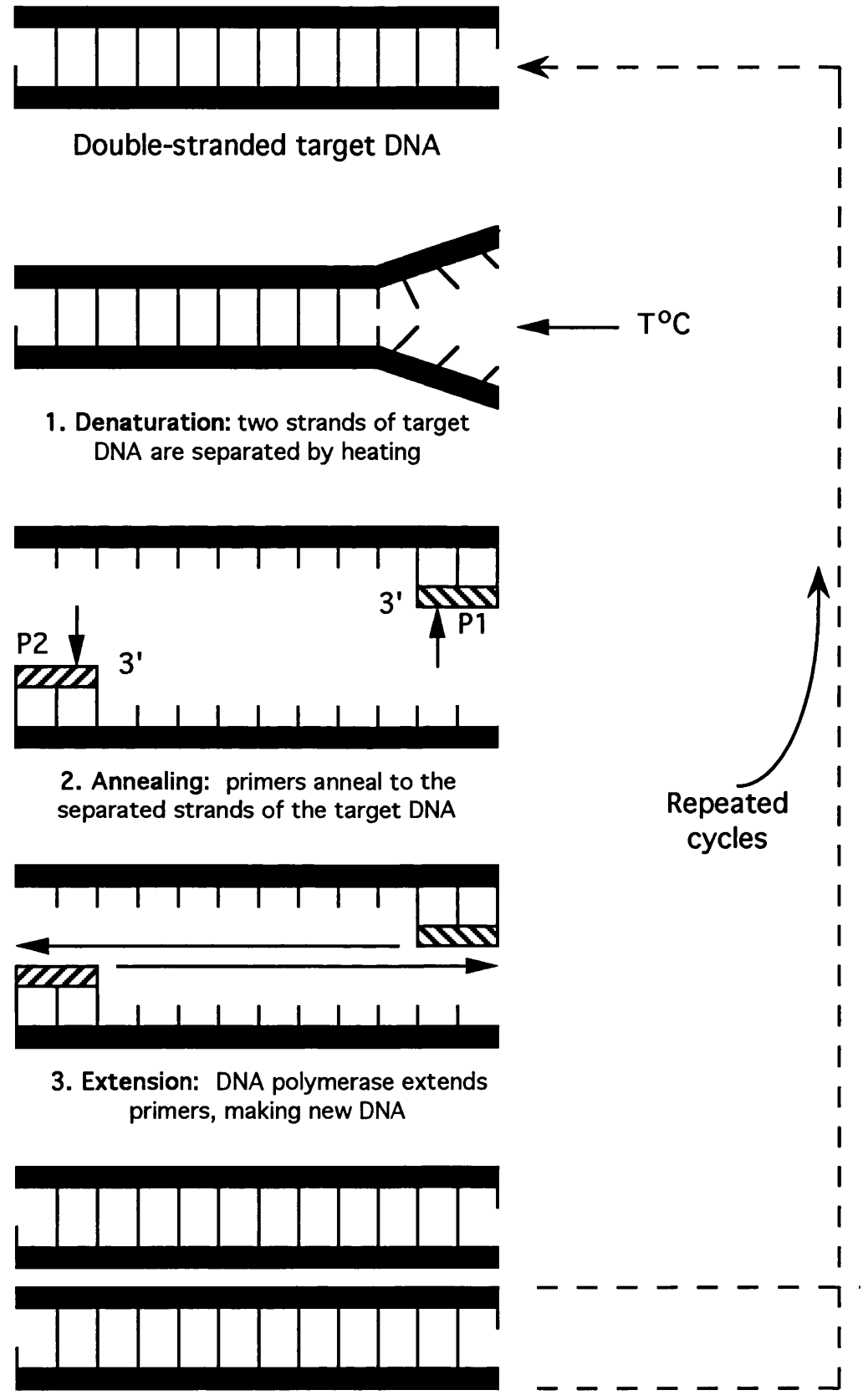


Figure 2. First cycle of DNA amplification by PCR.

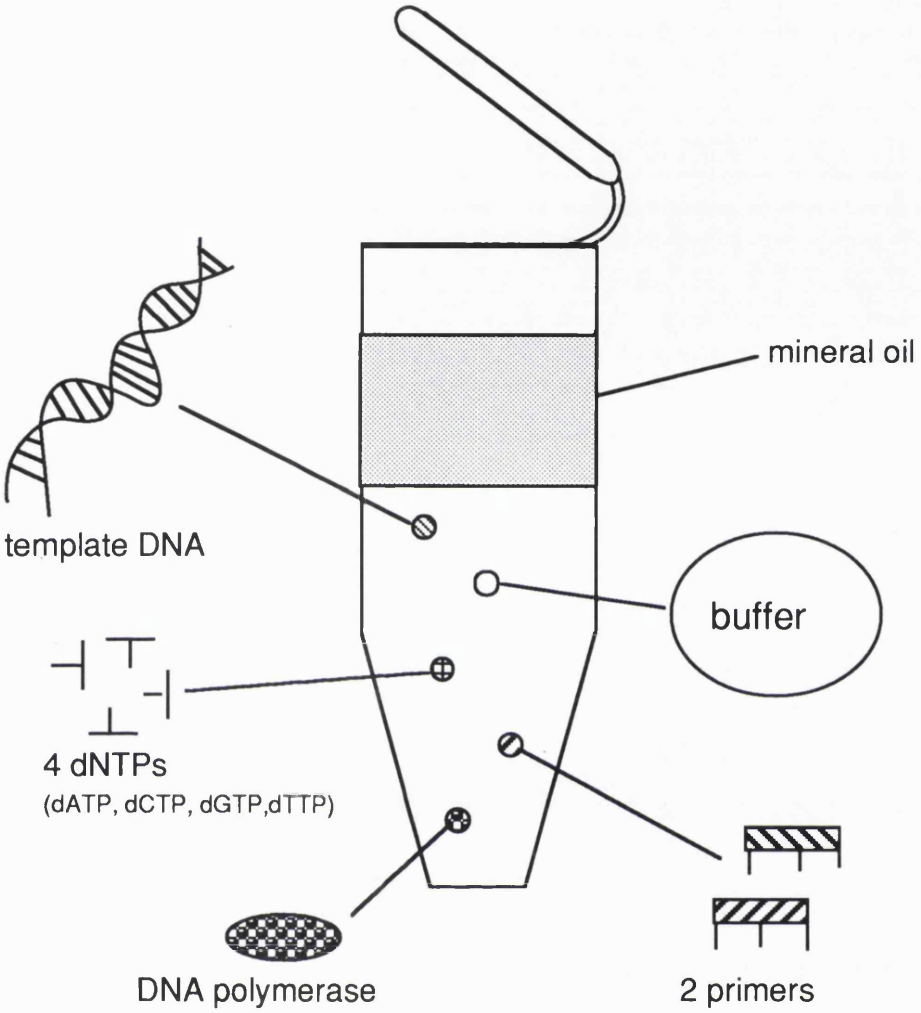


Figure 3. Components of PCR.

PCR buffer. The standard buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 100 μg/ml of gelatin is adequate for the majority of genomic PCRs. Depending on the particular primer pair used, changing the $MgCl_2$ concentration from 1 to 10 mM can have dramatic effects on the specificity and yield of an amplification. Further, the reduction or elimination of KCl and gelatin may also improve the performance of the reaction (Innis *et al.*, 1988).

Primers. The selection of efficient and specific primers is somewhat empiric. There are no distinct rules that guarantee the choice of an effective primer pair. Fortunately, the majority of primers can be made to work, and the following guidelines may help in the design and selection of primers:

1. Where possible, primers should be selected with an average G+C content of around 50% and a random base distribution. Primers with stretches of polypurines, polypyrimidines, or other unusual sequences should be avoided if possible.

2. Sequences with significant secondary structure should also be avoided. Computer programmes are available which are very useful for revealing these structures.

3. Primers should be checked against each other for complementarity.

Most primers will be between 20 and 30 bases in length, and the

optimal amount used in an amplification will vary. In general, concentrations ranging from 0.1 to 0.5 μM of each primer should prove acceptable (Innis *et al.*, 1990).

Deoxynucleotide triphosphates (dNTPs). dNTPs (dATP, dCTP, dGTP, and dTTP) are usually present at 50 to 200 μM of each and should be balanced (i.e., all four at the same concentration). At these concentrations, there is sufficient precursor to synthesize about 6.5 to 25 μg of DNA (Innis *et al.*, 1990).

Taq DNA polymerase. The optimal concentration for Taq DNA polymerase is about 2 units/100-μl reaction. Concentrations in excess of 4 units tend to result in the accumulation of nonspecific amplification products, whereas amounts less than 1 unit usually reduce the yield (Innis *et al.*, 1990).

Template. In PCR, the target of amplification can be DNA or RNA. If an RNA sequence is to be amplified, a DNA copy of it (cDNA) must be synthesized by using reverse transcriptase before the PCR is begun (Schochetman *et al.*, 1988). Thus the template of amplification in PCR may be DNA or cDNA. The target does not have to be pure DNA, material can be used directly from laboratory, clinical, ancient, or any other kind of specimens.

Oil overlay. Most thermal cyclers do not heat the lids of the reaction tubes. Therefore, an overlaying of the amplification mix with light mineral oil is necessary to prevent evaporation. Any evaporation would lead to higher reagent concentration and to a decrease in temperature (Mezei, 1990). In addition, the oil helps to prevent cross-contamination. But, too much oil slows the thermal process. Standardizing the amount of oil overlay minimizes these factors. The type of thermocycler determines how much oil is needed to achieve the best compromise. In thermal cyclers, where the lid of the reaction tube is heated continuously to >96°C, no oil overlay is necessary. For most applications using other cycles, 70 µl oil for 100 µl reactions, 40 µl oil for 50 µl reactions, and 30 µl oil for 25 µl reactions is generally sufficient (Rolfs *et al.*, 1992).

General rules for PCR set-up (Rolfs *et al.*, 1992):

1. Sources of contamination with extraneous DNA must be avoided.
2. Physically separated areas for pre-PCR and post-PCR procedures are necessary, e.g., laminar flow hood, separated rooms.
3. Personal reagent sets and pipettes should be used.
4. Freshly washed laboratory coats should be used each day.
5. Disposable gloves must be worn and they should be changed frequently.

6. Disposable materials, bottles and tubes must be used.

7. Prelabelled tubes and thawed reagents should be placed on ice.

8. Different sets of pipettes for reagents and DNA samples must be used.

9. The thermal cycler must be preheated.

Different varieties of PCR. In addition to standard PCR, it is now possible to perform different types of PCR in terms of different requirements. The applications of these PCRs have promoted basic molecular biology research as well as diagnostic medicine. Some of these methods include the following:

Asymmetric PCR, Competitive PCR, Differential PCR, Expression PCR, *In-situ* PCR, Inverse PCR, Multiplex PCR, Nested-primer PCR, Reverse-transcription PCR (RT-PCR).

PCR and medical microbiology. PCR has revolutionized the detection of bacterial, viral, and fungal pathogens. The targeted amplification of nucleic acid sequences provides not only dramatic increases in the number of copies to be detected but concomitantly provides a nearly equivalent reduction in the complexity of the nucleic acid to be probed. Either DNA or RNA can be used as a template for amplification and even needle punch biopsies and aspirates of various types provide sufficient material for PCR analysis. This method has

immense potential in the sensitive, specific, and rapid diagnosis of infection (Schochetman, 1988; Persing and Landry, 1989; Eisenstein, 1990). The whole process of PCR can be completed in a few hours, making the same-day diagnosis of infection possible.

2.4. Aims of the thesis

The main aims of the study described in this thesis were:

1) to find out whether leprosy PCR detects *M. leprae* in appropriate samples that are missed by microscopy.

2) to find out whether tuberculosis PCR identifies the presence of *M. tuberculosis* missed by direct microscopy and culture.

3) to discover whether the serological methods can distinguish between long-term-treated leprosy patients positive by PCR from those negative by PCR.

4) to relate the findings to the skin test and immunotherapy data.

PART TWO:

DEVELOPMENT OF THE STUDY

CHAPTER 3

State of the Study, Patients and Clinical Specimens

3.1. Introduction

The available information about leprosy in Iran (former Persia), is very scanty and limited. Obviously, rarity of investigations on leprosy and its situation in Iran, has caused this condition. Although it is believed that leprosy was brought back to Iran by troops of Darius and Xerxes from Egypt (Kohout *et al.*, 1973), the earliest documented information available showed its presence in some villages in the northwest (Azerbaijan), and northeast (Khorassan) of Iran but included the comment that "leprosy is not prevalent in Iran" (Gilmour, 1925).

At present, there are three leprosy sanatoriums in three different parts of Iran that admit leprosy cases from throughout the country. The oldest and main one is Baba Baghi Leprosy Sanatorium on which the present study was based. In fact, Baba Baghi was a small village that has gradually changed into a sanatorium over the years. It was especially suitable for an old-style leprosarium surrounded with high and rocky mountains, and an enclosed area of suitable land for farming. A documentary investigation showed that this leprosarium has been functioning for about 100 years (Nasseri and Ko, 1977) and was

moved from a neighbouring valley to the present site some 50 years ago. Baba Baghi village with a population of under 2000, is located 25 Km northwest of the mountainous city of Tabriz, the capital of the Eastern Azerbaijan province and one of the four major cities of Iran. It is situated in the northwest of the country, near the former Soviet Union (U.S.S.R.) and Turkish borders. The climate of the region is affected by the Siberian climate. It is dry with very cold winters and mild summers. The majority of leprosy patients residing in Baba Baghi are old patients who have married amongst themselves and sometimes have large families of healthy children. The major occupation of the residents is farming. There is a hospital in the sanatorium which has been led by a French medical team for many years; this admits the residents of the sanatorium when they have problems and also treats new leprosy cases. After diagnosis and confirmation, in the past, new cases of leprosy were compulsorily quarantined in the leprosarium hospital until completion of their necessary period of treatment depending on the type of leprosy although nowadays most are treated as out-patients. Multi-drug therapy (MDT) was introduced in Baba Baghi during the 1980s, but some patients living there who had apparently been cured after receiving dapsone monotherapy over many years were not given MDT. The survey of patients' records, shows that after treatment, most patients have preferred to stay and reside at Baba Baghi Sanatorium lifelong. Both sanatorium and hospital are supported

by the Iranian Government.

3.2. Individuals and specimens

In a preceding visit by Dr J.L. Stanford and colleagues to Baba Baghi Leprosy Sanatorium in 1991, 279 leprosy patients with long histories (more than 10 years) of treatment for leprosy, agreed to skin-testing with 4 new tuberculins: Tuberculin (T), Leprosin A (LA), Scrofulin (S), and Vaccin (V) (Shield *et al.*, 1977; Stanford *et al.*, 1989a; Ganapati *et al.*, 1989), and to be randomised to receive an injection of saline as placebo or killed *M. vaccae* as immunotherapy (IT) (Stanford *et al.*, 1990).

A year later in 1992, in the first sampling visit, my study began on selected groups of these patients (n=44). They had received dapsone monotherapy over many years and agreed to provide sputum and skin-tissue fluid specimens for PCR examination for tubercle and leprosy bacilli, and serum samples for antibody estimation. Although these selected patients seemed to have been bacteriologically cured, they still had physical signs and manifestations of the earlier active disease. Recent clinical examinations, however, showed no obvious symptoms of any relapse in their leprosy. Their ages were between 30 and 80 years, and according to their clinical records, over the years, 22 had initial

diagnoses of multibacillary (MB) leprosy, and 22 had initial diagnoses of paucibacillary (PB) leprosy. About half of them (21 patients) were selected as having Koch-type responses to tuberculin at skin-testing a year earlier suggestive of tuberculosis.

Eighteen months later, in the second sampling visit, repeat samples were obtained from 40 of the same selected patients, and an additional 10 patients were selected from the 279 in the immunotherapy study.

Eight non-tuberculous pulmonary disease patients were chosen as a control group to compare with the leprosy patients in the *M. tuberculosis* DNA study.

Serum samples from 15 healthy volunteers, 11 patients with active tuberculosis, and 3 active lepromatous leprosy (LL) patients were obtained as controls for the antibody studies.

The specimens were obtained from the patients as follows:

I. Skin-tissue fluid smears and swabs. These were taken from tissue fluid of old skin lesions of leprosy, and the chosen sites were cleaned with alcohol and allowed to dry. Next, the skin was pinched up into a fold between the index finger and thumb with enough pressure to stop or minimize bleeding. A cut of about 5 mm in length, and deep enough to penetrate into the infiltrated layer of the dermis, was made with a small sterile scalpel blade (Parker and Collier, 1990). Smears on microscope slides were prepared and skin-tissue fluid swabs were taken

(using E.N.T. swabs) from these cuts. After completion of collection, the skin-tissue fluid smears were examined microscopically in the laboratory and the skin-tissue fluid swabs were kept at -20°C for transport to London for PCR examinations.

II. Sputum samples. Sputa coughed up by the patients on waking in the morning, were collected and smears were prepared on glass slides of the concentrates prepared for culture. These were examined in the laboratory by direct microscopy and the remaining sputa were transferred to small screw-capped bottles, and kept at -20°C for transport to London for PCR examinations.

III. Serum samples. From each of the above mentioned subjects with the exception of one female patient of the selected group who refused to give blood, a 10 ml venous blood sample was obtained. The serum was separated carefully, and transferred to a small screw-capped bottle and then a crystal of merthiolate was added to the bottle, and labelled. All serum samples obtained from subjects were then kept at -20°C, and after completion of the collection, all of the sera were brought to London for serological examinations.

CHAPTER 4

Materials and Methods

4.1. Suppliers

BDH:

Acetone, citric acid, ethanol, H_2O_2 (20 volumes), H_2SO_4, HCl, KCl, $MgCl_2$, Na_2CO_3 (anhydrous), $Na_2HPO_4.2H_2O$, NaCl, $NaHCO_3$, NaOH, Nonidet P40

Bio-Rad:

Chelex 100 Resin

Baxter:

Sterile nonpyrogenic water

Dako:

IgG (γ-chains) (peroxidase-conjugated rabbit immunoglobulins to human), Streptavidin-HRP

Difco:

Lowenstein-Jensen (LJ) medium

Dynatech:

ELISA reader (Dynatech MR 5000)

Gibco:

Fungi-Bact

Hybaid:

Thermal cycler (Thermal-Reactor)

Intermed:

Nunc-Immunoplates Maxisorp F-96

Medical Wire & Equipment Co. LTD., (Corsham, Wilts., England):

E.N.T. swab

Oswel DNA Service (Edinburgh, UK):

S13 & S62 primers, IS*6110* primers

Perkin-Elmer:

Taq DNA polymerase

Pharmacia:

dATP, dCTP, dGTP, dTTP

Polaroid:

Polaroid film

Sigma:

ABTS (2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid]), boric acid, bromophenol blue, BSA (Albumin, Bovine), carbol fuchsin, diatomaceous earth, DNA Ladder (123 bp), EDTA, ethidium bromide, ϕX174 DNA *Hae*III digest, gelatin, glycerol, glycine, GuSCN (guanidine thiocyanate), KH_2PO_4, lauryl sulphate, merthiolate, methylene blue, mineral oil, *N*-acetyl-L-cysteine, $Na_2EDTA.2H_2O$, NaF, oil immersion, protein A, Tris-base, Tris-HCl, Triton X-100, Tween 20, xylene cyanol

Ultra-Violet Products, INC:

302-nm ultraviolet transilluminator

4.2. Buffers, reagents and solutions

10 X TBE:

1 litre distilled water

107.7 g Tris-base

55 g boric acid

7.45 g $Na_2EDTA.2H_2O$

ABTS solution:

25 ml CPB (pH 4.1)

12.5 mg ABTS

8.75 µl H_2O_2 (20 volumes)

Anti-GlcNAc solution:

This solution (biotinylated *N*-acetylglucosamine) was kindly supplied by Professor G.A.W. Rook.

Coating buffer:

1 litre sterile nonpyrogenic water

1.98 g Na_2CO_3 (anhydrous)

2.212 g $NaHCO_3$

CPB (pH 4.1):

21.02 g/litre 0.1 M citric acid (sol. A)

17.79 g/litre $Na_2HPO_4.2H_2O$ (sol. B)

Diatom suspension:

50 ml	distilled water
10 g	diatomaceous earth (celite)
500 µl	HCl (32% wt/vol)

Electrophoresis buffer:

270 ml	distilled water
30 ml	10 X TBE
15 µl	ethidium bromide

Gel-loading buffer:

0.25%	bromophenol blue
0.25%	xylene cyanol
30%	glycerol in H_2O

Glycine buffer (pH 7.0):

0.1 M	glycine
0.16 M	NaCl

Lysis buffer L6:

100 ml	0.1 M Tris-HCl (pH 6.4)
120 g	GuSCN
22 ml	0.2 M EDTA (pH 8.0)
2.6 g	Triton X-100

Mycobacterial antigen reagents:

Sonicated *M. tuberculosis* (son. tb), secreted *M. tuberculosis* (sec. tb), sonicated *M. vaccae* (son. vacc), and sonicated *M. fortuitum* (son. fortu) antigens were kindly supplied by Dr J.L. Stanford, UCLMS, London, UK. Sonicated *M. leprae* (son. lepr) was generously provided by Dr M.J. Colston and PGL-I (armadillo derived PGL-I) specific antigen of *M. leprae* was kindly donated by Dr P. Draper both from National Institute for Medical Research (NIMR), Mill Hill, London, UK. The 65 kDa heat shock protein (hsp) was generously supplied by Dr M. Singh of GBF, Braunschweig, Germany from WHO sources.

PBS (pH 7.4):

1 litre	distilled water
8 g	NaCl
0.2 g	KH_2PO_4
1.135 g	$Na_2HPO_4.2H_2O$
0.2 g	KCl

PBS-Tween 20 (pH 7.4):

1 litre	distilled water
8 g	NaCl
0.2 g	KH_2PO_4

1.135 g	$Na_2HPO_4.2H_2O$
0.2 g	KCl
0.5 ml	Tween 20

PCR gel:

45 ml	distilled water
0.5 g	agarose
5 ml	10 x TBE
2.5 µl	ethidium bromide

PCR buffer:

500 mM	KCl
100 mM	Tris-HCl (pH 8.3)
15 mM	$MgCl_2$
0.1%	gelatin

Protein A solution (2.5 µg/ml):

6 ml	PBS (pH 7.4)
7.5 µl	protein A

Streptavidin solution (1 µg/ml):

4 ml	PBS-Tween 20-1% BSA
1 µl	streptavidin-HRP

TE buffer:

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

Washing buffer L2:

100 ml 0.1 M Tris-HCl (pH 6.4)

120 g GuSCN

4.3. Acid-fast microscopy (AFM).

Sputum samples and skin-tissue fluid specimens were prepared, fixed, and stained by the Ziehl-Neelsen (ZN) method for acid-fast bacilli (AFB) using strong carbol fuchsin, acid-alcohol as a decolourant, and methylene blue as the counterstain. To demonstrate the leprosy bacilli, the ZN staining method was modified by the use of a weaker decolourizer than used for tubercle bacilli, and 5% H_2SO_4 instead of 20% H_2SO_4 in alcohol was used but 20% H_2SO_4 in alcohol was used to demonstrate the tubercle bacilli.

In the acid-fast staining procedure, the red dye carbol fuchsin was applied to a fixed smear, and the slide was gently heated for several minutes (heating enhances penetration and retention of the dye). Then

the slide was cooled and washed with water. The smear was next treated with acid-alcohol, a decolourizer, which removes the red stain from tissues and bacteria that are not acid-fast (The acid-fast microorganisms retain the red colour since the carbol fuchsin is more soluble in the cell wall waxes than in the acid-alcohol. In non-acid-fast bacteria, whose cell wall lack the waxy components, the carbol fuchsin is rapidly removed during decolourization, leaving the cell colourless). The smear was then stained with a methylene blue counterstain (Non-acid-fast cells appear blue after application of the counterstain) (Laidlaw, 1989).

After staining, more than 20 fields of each stained smear were examined carefully under the light microscope using an oil immersion (X100) lens.

4.4. Culture

For the tuberculosis study of leprosy patients, all collected sputum samples were treated with the protocol of *N*-acetyl-L-cysteine (NALC)-2% NaOH, inoculated onto Lowenstein-Jensen (LJ) media, and incubated at 37°C. The slopes were examined for growth every week for 6-8 weeks.

4.5. Procedures of DNA extraction from clinical specimens for PCR assays

Three different and simple DNA extraction methods for preparation of template DNA from samples were used as follows:

(1) DNA extraction by using guanidine thiocyanate and diatoms. This method was based on the lysing and nuclease-inactivating properties of guanidine thiocyanate (GuSCN) together with the nucleic acid-binding properties of diatoms. Diatoms are the fossilized cell walls of unicellular algae and consist almost entirely of silica allowing more disruption in diatom-nucleic acid networks by vortexing (Boom *et al.*, 1990; 1991).

For skin-tissue fluid swab specimens, each was placed in a 10 ml conical tube containing 1 ml phosphate buffered saline (PBS) at pH 7.4 plus a 2X concentration of Fungi-Bact (Innis *et al.*, 1990), and dispersed on a vortex-mixer. Next, the specimen was allowed to stand at room temperature overnight, after which it was again vortex-mixed.

In the case of sputum samples, each was liquefied as follows:
An equal volume of a 4% (wt/vol) solution of NaOH containing 1 g of *N*-acetyl-L-cysteine per 100 ml was added to a sputum sample, and the tube was periodically vortex-mixed for 20 min. The sample was made up to 20 ml phosphate buffer (67 mM Na_2HPO_4, 67 mM KH_2PO_4 at pH

6.8) and centrifuged at 3,000 X *g* for 20 min. The supernatant was decanted, and the resulting pellet was suspended in 1 ml of the same phosphate buffer (Victor *et al.*, 1992).

For subsequent DNA extraction, 50 µl of either type of treated specimen was prepared by the procedure described below.

Diatom suspension (40 µl) was mixed with 900 µl of lysis buffer L6 (GuSCN 120 g; 0.1 M Tris-HCl at pH 6.4, 100 ml; 0.2 M EDTA at pH 8.0, 22 ml; and Triton X-100, 2.6 g) in a 1.5 ml Eppendorf microcentrifuge tube, and briefly vortex-mixed. The clinical specimen (50 µl) was added to the above, vortex-mixed for 5 sec, and allowed to stand at room temperature for 10 min. Next, it was vortex-mixed again, and centrifuged at 12,000 X *g* for 15 sec. The supernatant was discarded, and the nucleic acid (NA)-pellet was washed twice with washing buffer L2 (GuSCN 120 g; and 0.1 M Tris-HCl at pH 6.4, 100 ml), twice with 70% ethanol and once with acetone. The acetone was removed and the NA-pellet was dried at 56°C for 10 min. Next, 100 µl of TE buffer (Tris-HCl at pH 8.0, 10 mM; and EDTA at pH 8.0, 1 mM) was added to the NA-pellet, vortex-mixed, and incubated for 10 min at 56°C. It was mixed again, centrifuged at 12,000 X *g* for 2 min, and 5 µl of the supernatant was used directly for PCR assay.

(2) DNA extraction by using Chelex^{R} 100. This procedure was based on the utilization of Chelex^{R} 100, a chelating ion exchange

resin. The alkalinity of Chelex^{R} suspensions and the exposure to 100°C temperatures result in disruption of the cell membranes and denaturation of the DNA, and can obtain a DNA solution suitable for PCR (Stein and Raoult, 1992).

After treating the skin-tissue fluid swab specimens, as mentioned above (in method 1), for subsequent DNA extraction by this method, 50 µl of the treated specimen was added to 200 µl of a suspension containing 20% Chelex^{R} 100 in a 0.1% Lauryl sulphate, 1% Nonidet P40, and 1% Tween 20 aqueous solution in a 1.5 ml Eppendorf microcentrifuge tube, and briefly vortex-mixed on a vortex-mixer. The mixture was then boiled for 10 min, centrifuged at 12,000 X *g* for 10 min, and 5 µl of the supernatant was used directly for PCR examination.

(3) DNA extraction by boiling. This method was based on heating the sample in a boiling water bath to break down the bacterial cell wall, and release the DNA (Kocagoz *et al.*, 1993).

After treating the skin-tissue fluid swab specimens, as described above (in method 1), 50 µl of treated specimen was added to 100 µl of TE buffer (10 mM Tris at pH 8.0, and 1 mM EDTA at pH 8.0) in a 1.5 ml Eppendorf microcentrifuge tube, and vortex-mixed. Next, it was placed in a boiling water bath for 10 min, and then 5 µl of the suspension was used directly for PCR examination.

4.6. Preparation of *M. leprae* chromosomal DNA

M. leprae DNA was isolated from killed *M. leprae* (1.25 X 10^9 AFB/ml, killed by radiation) by all the three DNA extraction methods, as described above and was used as a positive control in PCR assays.

4.7. Preparation of *M. tuberculosis* chromosomal DNA

DNA of *Mycobacterium tuberculosis* was prepared and purified from a fresh culture of *M. tuberculosis* by a boiling method (McFadden *et al.*, 1990). A colony of *M. tuberculosis* was placed in 500 µl of sterile distilled water, boiled for 10 min, spun at 12,000 X *g* for 2 min, and 5 µl of the supernatant used as a positive control in every PCR assay.

4.8. Selection of primers for *M. leprae*

The primers used for the specific amplification of *M. leprae* DNA, were originally designed by Hartskeerl *et al.* (1989) and were selected on the basis of the nucleotide sequence of the gene encoding the 36 kDa

antigen of *M. leprae*. The primers were S13 and S62. The sequence of the primers which amplify a 530-bp fragment of the *M. leprae* DNA sequence, were:

S13 (5'-CTCCACCTGGACCGGCGAT-3') and

S62 (5'-GACTAGCCTGCCAAGTCG-3')

These primers were chosen because of their sensitivity, with a reported detection limit of approximately one bacterium. On the other hand, this set of primers had shown excellent results in amplification of *M. leprae* DNA particularly in clinical specimens when they had been compared with F (forward) and R (reverse) primers of Williams *et al.* (1990) which amplify a 360-bp fragment of the gene encoding the 18 kDa antigen of *M. leprae*.

4.9. Selection of primers for *M.tuberculosis*

The primers used for the specific amplification of *M. tuberculosis* were originally designed by Eisenach *et al.* (1990) from sequences which are repeated several times in the chromosome of *M. tuberculosis*. The sequences of the primers which amplify a 123-bp fragment of the repetitive DNA sequence of *M. tuberculosis* (IS*6110*) from 5' to 3' were:

CCTGCGAGCGTAGGCGTCGG and CTCGTCCAGCGCCGCTTCGG

These primers were chosen because of their sensitivity, with a detection limit of fewer than 10 bacilli. On the other hand, this set of primers had been successfully used in this laboratory.

4.10. PCR procedure

PCR were performed individually and separately for the DNA amplification of both *M. leprae* and *M. tuberculosis*. Five µl of each DNA extract were incubated in a 45 µl reaction mixture containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 1 µM each of primers (S13 and S62 for *M. leprae*; and IS*6110* primers for *M. tuberculosis*), 0.2 mM each of deoxynucleotides dATP, dCTP, dGTP and dTTP, and Taq DNA polymerase (2.5 units for *M. leprae* and 1.25 units for *M. tuberculosis*). The reaction mixtures were covered with 40 µl of sterile mineral oil to prevent evaporation. A control tube containing no target DNA (sterile distilled water prepared by either type of DNA extraction method as mentioned above) as a negative control, with another tube containing chromosomal DNA (of *M. leprae* or *M. tuberculosis*) as a positive control were included with every set of tests. Precautions were taken to avoid contamination with extraneous DNA as described in the general rules for PCR set-up (Chapter 2).

The reaction was performed using an automated thermal cycler (Hybaid Thermal Reactor) and for the amplification of *M. leprae* DNA, 45 amplification cycles were performed. Each cycle consisted of denaturation at 94°C for 2 min, annealing of primers at 55°C for 2 min, and primer extension at 72°C for 3 min. After the 45th cycle, the extension reaction was continued for another 12 min at 72°C (Hartskeerl *et al.*, 1989). For *M. tuberculosis* DNA amplification, the samples were denatured at 94°C for 5 min, and then 30 amplification cycles were done. Each cycle consisted of denaturation at 94°C for 2 min, annealing of primers at 68°C for 2 min, and primer extension at 72°C for 2 min. After the 30th cycle, the extension reaction was continued for another 5 min at 72°C (Eisenach *et al.*, 1990).

The presence of the amplification product (530-bp for *M. leprae* and 123-bp for *M. tuberculosis*) was sought by electrophoresis of 5 µl of the amplified mixture at 88 V for 45 min on an agarose gel (1%). The DNA was stained with ethidium bromide (0.5 µg/ml) and visualized on a 302-nm ultraviolet transilluminator (Hartskeerl *et al.*, 1989; Williams *et al.*, 1990; Eisenach *et al.*, 1990). The molecular size markers used were ϕX174 DNA *Hae*III digest for *M. leprae* and 123-bp DNA Ladder for *M. tuberculosis*.

In order to test for the presence of PCR inhibitors, PCR negative specimens were retested after adding 2 µl of the chromosomal DNA (of *M. leprae* and *M. tuberculosis*) to the amplification mixtures. These

were amplified again as described above. The specimen was considered to be inhibitory if no amplification product was observed (De Wit *et al.*, 1991; Soini *et al.*, 1992).

Cross-contamination in PCR becomes evident when amplification occurs in negative controls. For preventing cross-contamination, two important points were always considered in addition to other precautions mentioned in Chapter 2:

1. Clean and sterile equipment and supplies were used.
2. The PCR set-up was done in three separated rooms, the first room for clinical sample preparation and DNA extraction (using a safety cabinet), the second room for making the reaction mixture and using the thermal cycler, and the third room for running gel and loading of amplified products.

4.11. Indirect ELISA procedure (Engvall and Perlmann, 1971; Nassau *et al.*, 1976)

ELISA plates (Nunc-Immunoplates Maxisorp F-96) were coated individually with sonicated *M. tuberculosis* (son. tb), secreted *M. tuberculosis* (sec. tb), sonicated *M. leprae* (son. lepr), PGL-I specific antigen of *M. leprae,* sonicated *M. vaccae* (son. vacc), sonicated *M. fortuitum* (son. fortu), and 65 kDa hsp antigens, diluted to 5 µg/ml in

coating buffer (pH 9.6), prepared in sterile nonpyrogenic water. To coat the ELISA plates, 50 µl of antigen reagent was added to each well, and incubated overnight at 4°C in a humid environment.

The next day, ELISA plates were washed 3 times with PBS (pH 7.4)-Tween 20, and blocked at 37°C with 50 µl of PBS-Tween 20 containing 1% of bovine serum albumin (BSA) (in sterile nonpyrogenic water) in each well for 2 hours. They were washed again 3 times with PBS-Tween 20. Then serum samples, diluted 1:1000 with PBS-Tween 20-1% BSA (the serum dilution had been optimized to 1:1000 IgG estimations), were added in 50 µl volumes to duplicate wells. Next, ELISA plates were incubated at 37°C for 2 hours in a humid environment, and again washed 3 times with PBS-Tween 20-1% BSA. Then peroxidase-conjugated rabbit antiserum to human IgG gamma chains was added at dilution of 1:2000, 50 µl/well, and the ELISA plates were incubated overnight at 4°C in a humid environment.

On the last day, the ELISA plates were washed again 3 times and the reaction was developed by adding 50 µl of ABTS (2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid]) substrate dissolved in citrate-phosphate buffer (CPB) (pH 4.1) and hydrogen peroxide solution (20 volumes) added just before use, to each well. After incubation of the plates in the dark for 30 min at 37°C in a humid environment, the enzymatic reaction was stopped by adding 50 µl of 0.2% sodium fluoride solution to each well. The optical density was read at 630 nm

using an ELISA reader (Dynatech MR 5000).

Sensitivity and specificity results were calculated using the following formulae:

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

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

The cut-off value for ELISA was calculated from the absorbance values from the healthy controls by adding 2 standard deviations to the mean value (Vikerfors *et al.*, 1993).

4.12. Agalactosyl IgG (G_0) assay procedure (Rook *et al.*, 1991a)

ELISA plates (Nunc-Immunoplates Maxisorp F-96) were coated with 2.5 µg/ml of protein A in PBS by adding 50 µl/well and incubating overnight at 4°C in a humid environment.

Next day, the excess protein A was poured off and 100 µl of PBS-Tween 20-1% BSA was added to each well. This was incubated for an hour at 37°C in a humid environment. The plates were then washed 3 times with PBS-Tween 20. Serum samples were diluted 1:100 with glycine buffer (0.1 M glycine and 0.16 M NaCl adjusted to pH 7.0 with

NaOH) to reduce IgG aggregation. Next, 50 µl/well of the diluted sera were added to duplicate wells and plates were incubated for 2 hours at 37°C. A set of standard human sera of which the G_0 value had been determined, were included in each assay. The plates were washed as previously described with the last wash containing only PBS (without Tween 20 or BSA). Next, 50 µl/well of PBS at pH 7.2 was added and plates were carefully placed on the surface of water in a 85°C water bath for 7-8 min. This was to denature the bound IgG and hence expose the relevant sugars. The plates were allowed to cool for a few minutes. The PBS was poured off and plates were dried. Next, 50 µl/well of anti-GlcNAc solution at a concentration of 2 µg/ml in PBS-Tween 20-1% BSA, was added and plates were incubated overnight at 4°C in a humid environment. This anti-GlcNAc solution was a biotinylated N-acetylglucosamine, a monoclonal antibody produced by immunising mice with a group A streptococcal cell wall peptidoglycan/polysaccharide mixture which was selected for its specificity to terminal N-acetylglucosamine, GlcNAc.

On the last day, plates were washed again 3 times with PBS-Tween 20 and 50 µl/well of peroxidase-conjugated streptavidin, at a concentration of 1 µg/ml in PBS-Tween 20-1% BSA, was added. The plates were then incubated at 37°C for an hour in a humid environment. Next, the plates were washed again 3 times with PBS-Tween 20, and 50 µl/well of ABTS substrate dissolved in CPB (pH 4.1) and H_2O_2 (20

and H_2O_2 (20 volumes) (prepared just before use) was added. After the immediate incubation of the plates in the dark for 15-20 min at 37°C in a humid environment, the enzymatic reaction was stopped by adding 50 µl of 0.2% NaF solution to each well. The optical density (OD) was measured at 630 nm using an ELISA reader (Dynatech MR 5000).

To plot the ODs of the standard sera, a curve fitting programme (Dataplot by S.M. Fraser, Strathclyde University, Glasgow, UK) was used. The results were calculated by log-linear interpolation of test ODs against the standard OD corrected for inter-plate variation. The final $\%G_0$ obtained was corrected for age. Percentages of G_0 less than 10 were considered as normal in all ages of tested individuals (Parekh *et al.*, 1988).

4. 13. Statistical analysis of results

Where appropriate Fisher's exact test and Student's t test were used to determine the likely statistical significance of my findings.

CHAPTER 5

DNA Extraction and Use of PCR to Detect *M. leprae* DNA

5.1. Introduction

Leprosy (Hansen's disease) is a chronic mycobacterial disease, infectious in some cases, which still afflicts millions of people worldwide. *M. leprae*, the aetiologic agent of leprosy, is one of the human pathogens which cannot be grown *in vitro* (Parker and Collier, 1990). The long incubation period, together with the wide spectrum of clinical manifestations of leprosy, have prevented reliable and rapid diagnosis of infections, especially in tuberculoid and indeterminate forms of leprosy (WHO, 1988). In the absence of any possibility of the cultivation of *M. leprae*, studies on the presence of *M. leprae* in the patients' specimens are based exclusively on Ziehl-Neelsen staining, with the inevitable shortcomings of lack of sensitivity and specificity.

Fortunately, new molecular methods have been developed as reliable and sensitive diagnostic tools for the identification of leprosy bacilli. The most significant advance in useful molecular methods, applicable to diagnosis, has been the polymerase chain reaction (PCR). The polymerase chain reaction amplifying a fragment of DNA replaces to a great extent the cultivation of microorganisms.

One of the remaining issues regarding the use of PCR technique is the extraction of DNA from clinical samples prior to performance of PCR. In this study, three different DNA extraction methods for preparation of template DNA were tested, and compared in order to select the best method for using in the future studies.

As mentioned in Chapter 3, in spite of introducing MDT in Baba Baghi Leprosarium during the 1980s, some patients residing there who had apparently been cured after receiving dapsone monotherapy over many years were not given MDT. It was of interest for many purposes to monitor the bacteriological status of such patients by using the more sensitive molecular methods now available.

In the study described here, a standard PCR was performed to detect specific *M. leprae* DNA in specimens from these "fully treated" long-term leprosy patients using the primers for a 530-bp fragment of the gene encoding the 36 kDa antigen of *M. leprae*, with a detection limit of approximately one bacterium (Hartskeerl *et al.*, 1989).

The main aim of this investigation was to find out whether PCR detects bacilli that are missed by microscopy, the presence of which might herald relapses in clinical disease, and to choose a reliable method for the small-scale purification of nucleic acid (NA) from clinical specimens.

5.2. Results

The amplification of the 530-bp fragment of *M. leprae* DNA by PCR, after preparation of the template DNA (using chromosomal DNA) by three different methods, is shown in Figure 4. A stronger band was produced by PCR amplification when bacterial DNA was obtained by the method using guanidine thiocyanate (GuSCN) and diatoms compared with the two other DNA extraction methods. The weaker band was produced by PCR amplification when bacterial DNA was extracted by the heating procedure.

44 skin-tissue fluid swab specimens prepared by all the three DNA extraction procedures, were tested by PCR (Table 4). Ten of the above specimens (22.7%) were found to be PCR-positive when prepared by the GuSCN-diatoms procedure, whilst five of the same (11.4%) specimens were PCR-positive when prepared by the Chelex^{R} 100 procedure, but only one of the same (2.3%) specimen was PCR-positive when prepared by the heating procedure. It was concluded that the most promising results were obtained by the GuSCN-diatoms procedure, so this was chosen as a reliable DNA extraction method to use for the preparation of sputum samples.

A total of 88 clinical specimens from 44 patients were examined under the microscope after staining for AFB and none were seen. In contrast, PCR detected the presence of the 530-bp DNA fragment

specific for leprosy bacilli in 12 (13.6%) of the specimens when they prepared by the GuSCN-diatoms procedure. No PCR inhibitors were found in any of the PCR negative samples. Ten of the 44 skin-tissue fluid swabs were positive and 2 of the 44 sputum samples were positive by PCR. One set of PCR results is shown in Figure 5. Table 5 compares the results of PCR with acid fast microscopy (AFM). In one patient PCR was positive for both skin-tissue fluid and sputum, thus material from *M. leprae* was detected in a total of 11/44 patients.

Amongst male patients, 7/31 were PCR positive (including the patient positive in both samples), and amongst females, 4/13 were positive.

The distribution of the PCR results according to type of the original disease, is shown in Table 6. The positivity rate of PCR for paucibacillary (PB) patients (36.4%) was about 3 times higher than in multibacillary (MB) patients (13.6%).

The distribution of the PCR results according to age of patients is shown in Table 7. The greatest positivity rate was found in the youngest age group of those aged between 30 and 45 years, although this group was very small.

Correlation between PCR-positivity of leprosy and skin test positivity to four new tuberculins tested a year earlier are summarised in Table 8. The greatest correlation was found between PCR-positivity of leprosy and skin test positivity to leprosin A.

Table 9 shows that the PCR-positivity rate of leprosy examined a year later, was significantly different between patients who had received immunotherapy with *M. vaccae* (2/23, 8.7%) and those who had received placebo with saline (9/21, 42.9%) ($p < 0.01$).

In total, 25% of the "fully treated" long-term leprosy patients in this study, were found to be PCR-positive for *M. leprae* by this technique.

Regarding repeat samples from the second visit, 6 of the same previous 10 PCR-positive skin-tissue fluid specimens were PCR-positive for leprosy again and the 34 remaining were found to be PCR-negative for leprosy, whereas all of the sputum samples from the second visit were found to be PCR-negative for leprosy. All of the 80 repeat samples were also negative for AFB. With regard to samples from an additional 10 randomly-selected patients (who were not in the second part of the study before), which were collected in parallel with repeat samples of the second visit, all were negative by AFM and PCR. PCR inhibitors were not found in any of the PCR-negative samples from the second visit.

5.3. Discussion

The primers S13 and S62, were chosen on the basis of their selecting a specific nucleotide sequence of a gene encoding the 36 kDa antigen of

M. leprae, which can then be amplified. The significant specificity of this set of primers means that they give no amplification of human DNA or of DNA from a number of other bacteria which may be present in human derived samples (Hartskeerl *et al.*, 1989). Thus this method is highly useful for the accurate detection of *M. leprae* DNA in human specimens. The detection limit of this PCR with primers S13 and S62, is reported to be 1 to 10 bacilli (Hartskeerl *et al.*, 1989), and recently, *M. leprae* DNA from an ancient bone dating from 600 AD, has been identified by their use (Rafi *et al.*, 1994). Theoretically this technique should be much more sensitive than other methods, such as microscopy for the direct detection of *M. leprae* .

In this study, three different DNA extraction procedures were tested, the results showed that the guanidine thiocyanate (GuSCN)-diatoms procedure of DNA extraction has the highest efficiency (Table 4). Chelex^{R} 100 had only half the efficiency in DNA extraction but the time of preparation of clinical specimens for PCR analysis was dramatically reduced and the number of manipulation steps could be minimized to decrease cross-contamination and contamination from extraneous DNA. A DNA solution for PCR examination could be obtained within 25 minutes by this method. Although the heating method is simpler than the other methods, it is not recommended because of its low efficiency. Other methods for DNA extraction from clinical samples have been reported to be successful, but some of them

(Pattyn *et al.*, 1993; De Wit *et al.*, 1993; Wichitwechkarn *et al.*, 1995) remain time-consuming and too laborious, so would be impractical for routine use in most clinical laboratories.

In view of the fact that the detection limit of the PCR performed in this investigation is 1 to 10 bacilli as mentioned before, it is believed that application of the GuSCN-diatoms method of DNA extraction in this PCR can provide a simple, rapid, and reliable technique for the identification of *M. leprae* DNA in clinical specimens. Because *M. leprae* has a very hard-to-digest cell wall, it is believed that the application of the GuSCN-diatoms method will be very helpful for DNA extraction of other bacteria including other mycobacteria from clinical specimens.

The finding that 11/44 "fully treated" long-term leprosy patients were still positive by PCR for *M. leprae*-specific DNA is surprising since all the patients in this study were thought to have been cured by dapsone monotherapy before 1982. Although it is possible that the bacilli from which the DNA was amplified were dead, and the data (Rafi *et al.*, 1994) on its survival in ancient bone raises this possibility, it would be surprising if such long pieces of DNA could survive in living host tissues for long after the bacilli were killed and even their acid fast ghosts had disappeared. The greater PCR-positivity of patients with paucibacillary (PB) disease over those with multibacillary (MB) leprosy also makes it unlikely that the DNA came from dead bacilli,

since numbers of these might be expected to be much greater in the latter patients.

Detection of DNA of *M. leprae* in skin-tissue fluid swabs really does suggest that live bacilli are still present, which might be as persisters, held in a state of virtually suspended animation by the immune mechanisms of the host. A study reported by WHO (1982) showed that in a small proportion of patients properly treated with dapsone monotherapy, persisters may survive for as long as 20 years. They might also be vegetative organisms about to lead to relapse. It is more difficult to be sure of the significance of positive results on sputum samples, since these could more readily be from external contamination.

The surprise finding, of more positive results on skin-tissue fluid swabs from patients who had paucibacillary disease (8/22) than from those who had multibacillary disease (2/22), reaches statistical significance ($p < 0.03$) (Table 6). An obvious explanation for this is the likely difference in treatment given to those with the two types of disease. Whereas multibacillary leprosy was treated with several years of high dose dapsone, paucibacillary patients often were given shorter treatment with low dose dapsone. Once a paucibacillary patient has apparently overcome his infection, chemotherapy can usually be stopped with impunity, since the active disease rarely recurs. Late reactions of reversal type occurring in a proportion of such patients

are put down to residual immunological responses to remaining antigen; the results of this study suggest a different explanation. A correlation might be sought between the occurrence of late reactions in PB patients and the detection of *M. leprae* DNA in their tissues.

In reality the result of a short course of dapsone for PB disease may be to slow the metabolic activity of the bacilli and perhaps kill a proportion of them, thus allowing the immune system to regain control of the situation and returning the infection to a latent form in which the bacilli continue as persisters in some patients. This would not occur in the treatment of MB disease, where the patient seems to lack the immunological power to hold the organisms in a persister state, and all have to be killed by the chemotherapy to stop reactivation from occurring. Such patients, after cessation of chemotherapy, may return to a state of high susceptibility to re-infection. The 2/22 MB patients found to be PCR-positive on skin-tissue fluid specimens in this study may be re-infected patients progressing towards a relapse of their disease.

No considerable correlation was found between sex and PCR-positivity of leprosy, but the groups were of very different size.

Table 7 shows that younger patients are more likely to have detectable DNA of *M. leprae* in their tissues than are older patients. This may be due to age, but might also be due to the preponderance of PB disease in the younger group which is suggested by the data.

Compounds in biological material, such as haemoglobin, can inhibit the amplification reaction (Innis *et al.*, 1990; Victor *et al.*, 1992) and it would be reasonable to minimize or remove them before DNA amplification can be done reliably, so if no amplified product is seen, inhibition is likely and the sample may require dilution or further purification. Fortunately, PCR inhibitors were not observed in this study. An explanation for this may be the use of diluted samples in the PCR study, as this minimizes the presence of PCR inhibitors (Folgueira *et al.* 1993).

The correlation between PCR-positivity of leprosy and skin test positivity to four new tuberculins and effectiveness of the immunotherapy of leprosy with killed *M. vaccae* are discussed in Chapter 9 (general discussion).

It seems that in this study, the results of the second sampling visit confirm the results of the first visit, because six of the same 11 Lepr-PCR positive patients of the first visit were found to be PCR-positive for leprosy on the second visit. In addition, of the five remaining negative patients, four were not available on the second visit and one had displayed a PCR-positive sputum sample, suggesting external contamination. The aim of selecting an additional 10 patients at random in the second sampling visit who displayed no positive results, was to increase the numbers in the selecting particularly of those with non-Koch response to tuberculin.

The results illustrate the potential value of PCR in the investigation of leprosy, and particularly indicate that PCR may be a useful tool for confirming that the bacterial load has actually been removed by treatment in PB disease. It would be interesting to extend the studies to patients receiving modern multi-drug therapy, and to discover whether the proportion of PB patients remaining PCR-positive after treatment includes those that suffer late-damaging reactions.

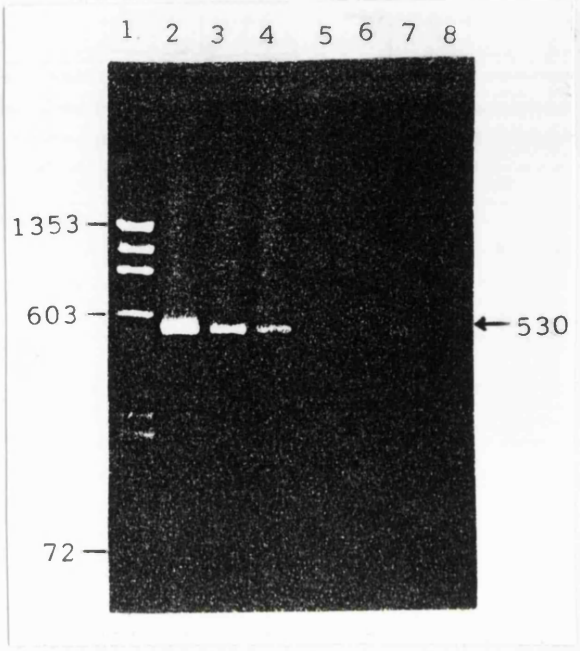


Figure 4. Comparison of the 530-bp amplification products by PCR from *M. leprae* chromosomal DNA extracted by three different methods. Lane 1, *Hae*III-digested phage ϕX174 DNA as a molecular size marker; lane 2, template DNA extracted by the method using guanidine thiocyanate and diatoms; lane 3, template DNA extracted by the Chelex^{R} 100 procedure; lane 4, template DNA extracted by the heating procedure; lane 5-7, no DNA (sterile distilled water prepared by either type of DNA extraction method respectively).

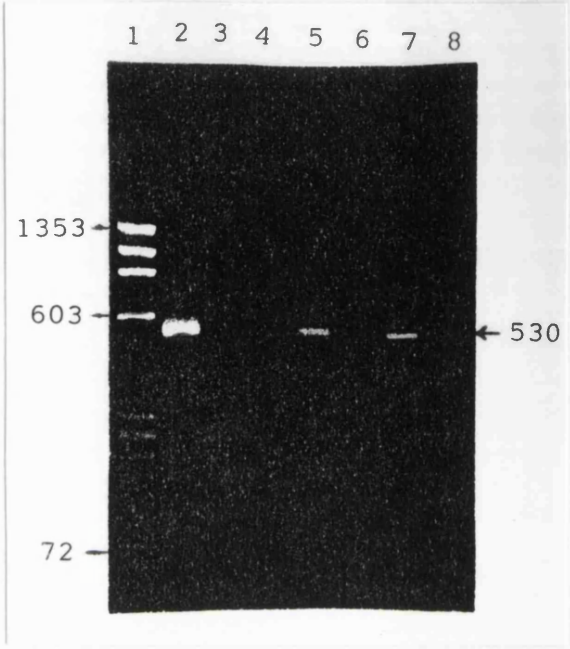


Figure 5. Typical results of PCR on a number of skin-tissue fluid specimens. Lane 1, molecular size marker; lane 2, *M. leprae* positive control; lane 3, negative control (no DNA); lanes 4, 6, and 8 are negative; lanes 5 and 7 are positive.

Table 4. Lepr-PCR results on skin-tissue fluid specimens using different DNA extraction procedures and comparative efficiency with the GuSCN-diatoms procedure.

Patient no.	Lepr-PCR results when using:		
	GuSCN & diatoms	Chelex	Heating
4	+	+	+
5	+	-	-
8	+	+	-
15	+	+	-
22	+	-	-
24	+	+	-
26	+	-	-
81	+	-	-
102	+	-	-
112	+	+	-
Total positives	10/10	5/10	1/10
Efficiency in comparison	100%	50%	10%

Table 5. Comparison of Lepr-PCR with acid fast microscopy (AFM) in clinical samples from long-term-treated leprosy patients.

Sample	No. tested	Positive	
		AFM	Lepr-PCR
Sputum	44	0 (0%)	2 (4.5%)
Skin-tissue fluid	44	0 (0%)	10 (22.7%)
Total	88	0 (0%)	12 (13.6%)

Table 6. Results of Lepr-PCR in long-term-treated leprosy patients according to type of leprosy.

*Type of leprosy	No. of patients	No. (%) of patients with positive results by Lepr-PCR			No. (%) of positive patients
		Sputum only	Skin-tissue fluid only	Both sputum & skin-tissue fluid	
MB	22	1 (4.5%)	1 (4.5%)	1 (4.5%)	3(13.6%)
PB	22	0 (0%)	8 (36.4%)	0 (0%)	8(36.4%)
Total	44	1 (2.3%)	9 (20.5%)	1 (2.3%)	11 (25%)

*MB, multibacillary leprosy
PB, paucibacillary leprosy

Table 7. Results of Lepr-PCR in long-term-treated leprosy patients according to age group.

Age group	No. of patients	No. (%) of patients with positive results by Lepr-PCR			No.(%) of positive patients
		Sputum only	Skin-tissue fluid only	Both sputum & skin-tissue fluid	
30-45	6	1 (16.7%)	2 (33.3%)	0 (0%)	3 (50%)
46-60	26	0 (0%)	6 (23.1%)	0 (0%)	6(23.1%)
61-75	9	0 (0%)	1 (11.1%)	0 (0%)	1(11.1%)
76 and over	3	0 (0%)	0 (0%)	1 (33.3%)	1(33.3%)
Total	44	1 (2.3%)	9 (20.5%)	1 (2.3%)	11(25%)

Table 8. Correlation between PCR-positivity of leprosy and skin-test positivity with 4 new tuberculins.

Skin-testing	No. of skin test positive* patients	No. (%) of Lepr-PCR positive patients amongst the skin test positive group (tested a year later)
Tuberculin	39	11 (28.2%)
Leprosin A	13	8 (61.5%)
Scrofulin	15	4 (26.7%)
Vaccin	14	5 (35.7%)

*Reactions of 2 mm or greater are taken as positive responses with these reagents (Stanford *et al.*, 1989; Ghazi Saidi et al., 1989).

Table 9. PCR-positivity rate of leprosy according to receiving immunotherapy (IT).

Receiving IT	No. of patients	No. (%) of Lepr-PCR positive patients (tested a year later)
M. vaccae (yes)	23	2 (8.7%) *
Saline (no)	21	9 (42.9%)
Total	44	11 (25%)

*$p < 0.01$

CHAPTER 6

Examination of Sputum Samples by PCR for *M. tuberculosis* DNA

6.1. Introduction

Tuberculosis and leprosy are among the most common disabling diseases in the world. It is estimated that around 50 million people have, or recently have had, clinical tuberculosis. More than 6 million people suffer from active leprosy with a similar number suffering from its resultant disability (Pallen, 1984). An association between tuberculosis and leprosy in individuals living in an area endemic for both diseases has been suggested, but little documented, although it is likely to be common. In some endemic areas the two diseases appear to coincide, whereas in others they seem mutually exclusive. Whether this is by chance, or reflects an interaction between the two diseases remains a matter of debate (Fine, 1984). It might be related also to the influence of immunity and susceptibility of variably distributed environmental mycobacteria. There is some evidence from the past that tuberculosis commonly afflicted institutionalised leprosy patients. One of the rare studies on this subject was done in 1895 by Armauer Hansen, who found tuberculosis to be the most common cause of death

among leprosy patients in Norway (Glaziou *et al.*, 1993). Recently, it has been shown that tuberculosis is no commoner in leprosy out-patients than in the general population (Jopling and McDougall, 1988), although two earlier studies suggested that leprosy patients are prone to tuberculosis. (Gatner *et al.*, 1980; Kumar *et al.*, 1982). The reality of the situation is probably that both diseases and the influence of environmental factors all interact, producing different sets of phenomena in different situations.

The present investigation was carried out as part of the follow-up of the same group of "fully treated" long-term leprosy patients in Baba Baghi Leprosy Sanatorium because about half of the patients had shown Koch-type responses to tuberculin suggestive of the possibility of tuberculosis. The diagnosis of tuberculosis in such patients is not always easy, and application of the polymerase chain reaction for this problem could be extremely useful. Recently, one quarter of this group of patients had been found to be PCR-positive for leprosy indicating relapse or re-infection in them (Chapter 5).

In this present study, a simple PCR was carried out to detect specific insertion sequences (IS*6110*) of *M. tuberculosis* DNA in sputum samples from such leprosy patients and also 8 non-tuberculous pulmonary disease patients (as the control group), using a set of primers with a detection limit of fewer than 10 bacilli (Eisenach *et al*, 1990; 1991). The GuSCN-diatoms method of DNA extraction was

chosen because its high efficiency had been demonstrated (Chapter 5). The aim was to search for tubercle bacilli by microscopy, culture, and PCR in sputum, and relate the findings to the skin test, immunotherapy data, and results of Lepr-PCR.

6.2. Results

Sputum samples obtained from 44 treated leprosy patients were tested by direct microscopy for AFB, and culture on LJ medium and none were found to be positive. In contrast, PCR detected the presence of the 123-bp DNA fragment specific for tubercle bacilli in 6 (13.6%) of the sputum samples. One set of PCR results from a number of sputum samples is shown in Figure 6. The results of direct microscopy, culture and PCR were negative in sputum samples from the 8 non-tuberculous pulmonary disease control group. No PCR inhibitors were detected in any of the PCR-negative specimens.

Comparison of the TB-PCR (tuberculosis PCR) results of this study with Lepr-PCR results of the previous study (Chapter 5) showed that none of the selected patients were found to be PCR-positive for both leprosy and tuberculosis concurrently.

Amongst male patients, 4/31 were TB-PCR positive, and amongst females, 2/13 were TB-PCR positive.

The distribution of the PCR results according to type of the original disease showed that there were equal numbers of TB-PCR positive results among patients with MB (3/22, 13.6%) or PB (3/22, 13.6%) diseases.

The distribution of the PCR results according to age of leprosy patients, apparently showed that the positivity rate of TB-PCR in older age group (51 years and over) (5/32, 15.6%) was higher than younger age group (1/12, 8.3%).

The correlation between PCR-positivity of tuberculosis and skin test positivity to four new tuberculins tested a year earlier is shown in Table 10. Apparently, T and LA gave similar results, with a slightly higher correlation with PCR-positivity of tuberculosis than S and V.

The results of PCR in relation to tuberculin testing are summarised in Table 11. Amongst patients with a Koch response to tuberculin, 5/21 (23.8%) were TB-PCR positive, in comparison with only 1/23 (4.3%) in those with non-Koch responses ($p = 0.074$).

There were equal numbers of TB-PCR positive results amongst patients who had received immunotherapy (3/23, 13%) or placebo (3/21, 14.3%) a year earlier (Table 12).

In total, 13.6% of the "fully treated" long-term leprosy patients in this investigation were found to be PCR-positive for *M. tuberculosis*.

With regard to repeat samples from the second visit, two of the same previous six TB-PCR positive sputum samples were found to be PCR-

positive for tuberculosis again, and the 38 remaining were found to be PCR-negative for tuberculosis. All of the 40 repeat sputum samples from the second visit were again negative for AFB and culture. Regarding sputum samples from the additional 10 randomly-selected patients collected in parallel with the repeat samples, all were found to be negative by AFM, culture, and PCR. None of the PCR-negative samples were found to be inhibitory.

6.3. Discussion

For the diagnosis of tuberculosis the standard is usually culture on selective media, however, the sensitivity of culture is difficult to judge (Daniel, 1990). The polymerase chain reaction (PCR) as a new diagnostic test, enables the specific detection of very small numbers of nucleic acid molecules of *M. tuberculosis*. The PCR used in this study shows the effectiveness of this technique for the rapid diagnosis of difficult cases of tuberculosis, considering the conventional methods gave negative results. The accuracy of this technique is demonstrated by the fact that the control group did not give any positive results.

Insertion sequences are apparently ideal targets for PCR amplification as multiple copies are usually present in the bacterial genome, and thereby provide a naturally occurring pre-amplification

step. One of these insertion sequences which is found only in the *M. tuberculosis* complex and can be used in the PCR protocol, is IS*6110* (Thierry *et al.*, 1989; Eisenach *et al.*, 1990). The repetitive nature of the target sequence of *M. tuberculosis* (IS*6110*) amplified by the PCR described here, probably contributes to the sensitivity, and as mentioned earlier, fewer than 10 bacilli can be detected by application of this PCR (Eisenach *et al.*, 1990). A recent study (Forbes and Hicks, 1993) has also strongly confirmed the ability of the IS*6110* primers to detect *M. tuberculosis* sequences directly in clinical specimens containing low numbers of tubercle bacilli.

It seems that the finding of 6/44 long-treated, admittedly selected, leprosy patients to be positive by PCR for *M. tuberculosis*-specific DNA is important, since one of the causes of death among leprosy patients, is tuberculosis (Jopling and McDougall, 1988). The finding of this investigation suggests that long-treated leprosy may encourage the development of tuberculosis. This suggestion is strengthened by the observation that none of the sputum samples from long-treated leprosy patients were found to have concurrent detectable *M. leprae* and *M. tuberculosis*. It is thought that the finding of this study differs from the few other studies (Gatner *et al.*, 1980; Kumar *et al.*, 1982; Glaziou *et al.*, 1993) on the association between leprosy and tuberculosis, as this present work relies on long-treated leprosy patients. Moreover, recently, a case of reactivation of tuberculosis and leprosy due to HIV

infection has been reported (Arora and Johri, 1994) suggesting this triple infection is increasingly likely to be diagnosed in endemic areas. The increased incidence of pulmonary tuberculosis observed in leprosaria, has given rise to the presumption that leprosy patients are susceptible to tuberculosis (Jopling and McDougall, 1988) without considering long-treated leprosy patients. One explanation for the association between long-treated leprosy and tuberculosis is that this is related to the distribution of environmental mycobacteria (Pallen, 1984) thus influencing immunity and susceptibility. Another possibility is that leprosy patients may share susceptibility factors to mycobacterial diseases with patients developing tuberculosis (Glaziou *et al.*, 1993). Long-term dapsone monotherapy of the patients of this study may be another factor in the development of tuberculosis amongst these leprosy patients. In contrast, multi-drug therapy (MDT) of leprosy includes rifampicin, one of the bactericidal antibiotics which can be effective against both *M. leprae* and *M. tuberculosis*.

Considering that patients with TB-smear positive sputa are the main sources of infection and TB-smear negative patients, whether TB-culture positive or not, are of very low infectivity (Parker and Collier, 1990), it is concluded that these six TB-PCR positive leprosy patients found in this study with smear and culture negative results, will have the lowest infectivity.

In this study, long-treated MB patients did not show a higher

proportion of TB-PCR positive results, in contrast to long-treated PB patients. An explanation for this finding may be the leprosy treatment of the patients of this study. It seems that both types of leprosy patients after treatment are at risk from tuberculosis in equal proportions. However, one study found a negative correlation between the tuberculoid form of leprosy and tuberculosis (Kaklamani *et al.*, 1991). A cause of this negative association could be a defective cellular immunity against mycobacteria in lepromatous patients, but this has never been demonstrated.

No important correlation was found between sex and PCR-positivity of tuberculosis, but as mentioned earlier, the groups were of very different size.

The distribution of the PCR results according to the age of leprosy patients, demonstrated that older leprosy patients were more likely to have detectable *M. tuberculosis* DNA in their sputum samples than were younger patients. This may be due to the decrease of the innate immunity in older patients, that is followed by the weakening of both external and internal defensive factors in older leprosy patients.

The correlation between PCR-positivity of tuberculosis and skin test positivity to four new tuberculins and effectiveness of the immunotherapy with killed *M. vaccae* are discussed in Chapter 9 (general discussion).

In the second sampling visit (done 18 months later) only two of the

same previous six TB-PCR positive patients were found to be PCR-positive for tuberculosis again. This might be due to failing in the collection of sputum samples from treated leprosy patients on the second occasion because it is so difficult to obtain sputum samples from such patients. Another possibility is that the leprosy patients suspected of tuberculosis might have received therapy in the sanatorium during the intervening period. However, the fact that two leprosy patients were found to be still TB-PCR positive in the second time, is of value. As mentioned in Chapter 5, the purpose of selecting an additional 10 patients at random in the second sampling visit who showed no positive results, was to increase the numbers in the selecting especially of those with non-Koch response to tuberculin.

The results indicate the usefulness of PCR as a valuable technique in the investigation of difficult cases of tuberculosis, and particularly suggest that it can be used to monitor the incidence of tuberculosis among "fully treated" long-term leprosy patients. It would be of considerable interest to extend this study to active leprosy patients, and those who have been treated with modern MDT, and find out whether PCR detects any suspected cases of tuberculosis amongst active leprosy patients.

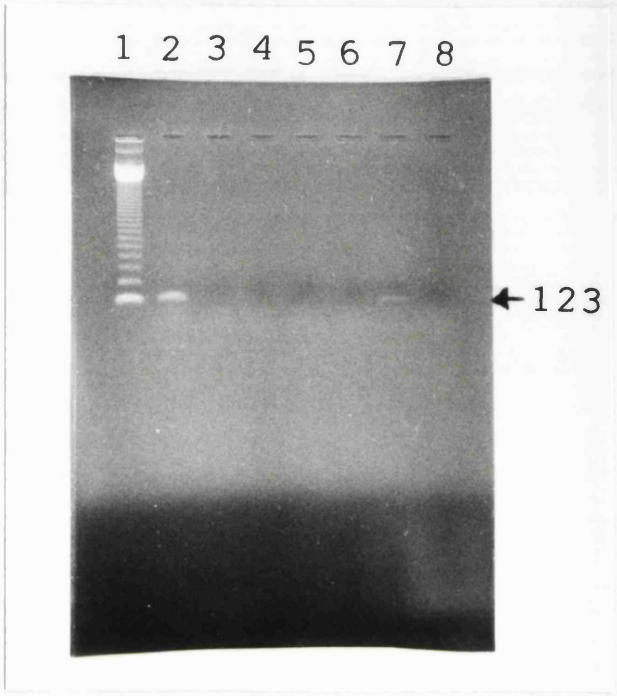


Figure 6. Typical PCR results from sputum samples. Lane 1, molecular size marker (123-bp DNA Ladder); lane 2, *M. tuberculosis* positive control; lane 3, negative control (no DNA); lanes 4-6 and 8 are negative; lane 7 is positive.

Table 10. Correlation between PCR-positivity of tuberculosis and skin-test positivity with 4 new tuberculins.

Skin-testing	No. of skin test positive* patients	No. (%) of TB-PCR positive patients among the skin test positive group (tested a year later)
Tuberculin	39	6 (15.4%)
Leprosin A	13	2 (15.4%)
Scrofulin	15	2 (13.3%)
Vaccin	14	2 (14.3%)

*Reactions of 2 mm or greater are taken as positive responses with these reagents.

Table 11. Results of TB-PCR in long-term-treated leprosy patients according to tuberculin testing.

Tuberculin testing	No. of treated leprosy patients	No. (%) of TB-PCR positive patients (tested a year later)
With Koch response	21	5 (23.8%) *
With non-Koch response	23	1 (4.3%)
Total	44	6 (13.6%)

* Not significant (p=0.074)

Table 12. Results of TB-PCR in long-term-treated leprosy patients according to receiving immunotherapy (IT).

Receiving IT	No. of patients	No. (%) of TB-PCR positive patients (tested a year later)
M. vaccae (yes)	23	3 (13%)
Saline (no)	21	3 (14.3%)
Total	44	6 (13.6%)

CHAPTER 7

A Study of IgG Response to Various Mycobacterial Antigens and Determination of the Level of Agalactosyl IgG

7.1. Introduction

Over the past 80 years, several serological tests have been developed to detect *M. leprae* infection (Melsom, 1983), however, the results have not been satisfactory. Interest in the past few years in serodiagnosis of leprosy has been heightened by the development of serological tests based on supposedly *M. leprae*-specific antigenic determinants (Brett *et al.*, 1983; Cho *et al.*, 1983; Young and Buchanan, 1983; Klatser *et al.*, 1985). The enzyme-linked immunosorbent assay (ELISA), one of the sensitive serological techniques (Wreghitt *et al.*, 1984) based on phenolic glycolipid-I (PGL-I), one of the two most prominent capsular lipids of *M. leprae* (Hunter and Brennan, 1981), is now widely used in serological studies of leprosy. The ELISA technique (Engvall and Perlmann, 1971) is based on the assumption that either an antibody or antigen can be coupled to an enzyme and resulting complex will retain both immunological and enzymatic activity. Indirect ELISA is suitable for the detection of IgG antibody, the main class of immunoglobulin in

serum that is the principal component of the secondary humoral immune response.

Tuberculosis is one of a group of diseases in which there is an increased percentage of circulating IgG molecules which lack terminal galactose from the oligosaccharides which are always present on the C_H2 domains of the Fc fragment of the IgG molecule. This glycoform of IgG is known as agalactosyl IgG (G_0) (Rademacher *et al.*, 1988). Raised levels of G_0 appears to be a marker of a type of immunoregulatory disturbance seen in several chronic T-cell mediated inflammatory diseases, including tuberculosis (Rook *et al.*, 1994). Agalactosyl IgG (G_0 = no galactose) is a glycoform of immunoglobulin G which lacks terminal galactose from the N-linked sugars (oligosaccharides) on the glycosylation site on the C_H2 domain of the Fc fragment of IgG. Apparently, reduced activity of galactosyltransferase (the enzyme that catalyses the addition of galactose to the oligosaccharide chains) in the B-cells, causes this situation (Parekh *et al.*, 1985; Axford *et al.*, 1987).

The present study was carried out as part of the follow-up of a group of long-term-treated leprosy patients one quarter of whom had been found to be PCR-positive for *M. leprae*. This was suggestive of continued infection, re-infection or relapse in such patients (Chapter 5) and a few of these patients (not the same as those PCR-positive for *M. leprae*) were found to be PCR-positive for *M. tuberculosis* indicating

infection with this organism (Chapter 6).

IgG antibody to six various mycobacterial antigen reagents was measured in serum samples from 43 long-term-treated leprosy patients and also 15 healthy volunteers, 11 patients with active tuberculosis, and three active lepromatous leprosy (LL) patients (as control groups) using an ELISA technique. The level of agalactosyl IgG was determined in sera from the same long-term-treated leprosy patients using a G_0 assay. The purpose of this study was to find out whether the ELISA technique could differentiate between long-term-treated leprosy patients positive or negative by PCR for *M. leprae*, and also whether the G_0 assay could be a valuable serological marker for tuberculosis in such patients positive by TB-PCR.

At the very last minute in my study the 65 kDa hsp of *M. tuberculosis* was obtained and tested with the sera from the long-term leprosy patients.

7.2. Results

All of the serum samples from individuals reacted to all antigens used in this study. The mean values of IgG antibody to various mycobacterial reagents in different groups are shown in Figures 7 and 8. The greatest reaction of IgG antibody to mycobacterial antigens was

found against sonicated and secreted *M. tuberculosis* antigens, whereas the lowest reaction was obtained with PGL-I antigen. Statistical comparison of the results demonstrated the healthy control group to be significantly different from other groups with all antigen reagents used in this investigation, with the exception of PGL-I. With PGL-I ELISA no statistically significant difference was found between the healthy control group and the active TB patients or TB-PCR positive patients, however, significant differences were found between active LL and active TB groups ($p < 0.001$). Differences were also found with PGL-I between Lepr-PCR positive and TB-PCR positive groups ($p < 0.02$), and Lepr-PCR positive and Lepr-PCR negative groups ($p < 0.001$). However, the difference between the two groups of TB-PCR positive and TB-PCR negative was not significant ($p < 0.2$) (Figure 9).

Figure 10 shows that in PGL-I ELISA amongst long-term-treated leprosy patients, 12/43 (27.9%) were found to be seropositive. Of the 12 seropositive patients in this group, nine were Lepr-PCR positive (75%) and the sensitivity and specificity of PGL-I ELISA for Lepr-PCR positive patients reached 81.8% and 90.6% respectively. Sensitivity and specificity of the other ELISAs for Lepr-PCR positive patients in this study were as follows:

1) Sonicated *M. leprae* (son. lepr) ELISA had a sensitivity of 100% and specificity of 15.6%.

2) Sonicated *M. tuberculosis* (son. tb) ELISA had a sensitivity of 45.5%

and specificity of 44.7%.

3) Secreted *M. tuberculosis* (sec. tb) ELISA had a sensitivity of 54.5% and specificity of 46.9%.

4) Sonicated *M. vaccae* (son. vacc) ELISA had a sensitivity of 100% and specificity of 46.9%.

5) Sonicated *M. fortuitum* (son. fortu) ELISA had a sensitivity of 100% and specificity of 50%.

Considering the high specificity of PGL-I ELISA in comparison with those for other antigen reagents, 9/11 (81.8%) of the Lepr-PCR positive patients found in *M. leprae* DNA study (Chapter 5) could be confirmed with PGL-I ELISA.

Amongst active LL patients, 3/3 (100%) were found to be seropositive for PGL-I whilst amongst active TB patients this ratio was 2/11 (18.2%), and in the healthy group was 1/15 (6.7%). In contrast, none of the TB-PCR positive patients of the *M. tuberculosis* DNA study (Chapter 6) were found to be seropositive for PGL-I (0/6, 0%).

Correlation between seropositivity for PGL-I and skin test positivity to 4 new tuberculins tested a year earlier are shown in Table 13. Most correlation was obtained between seropositivity for PGL-I and skin test positivity to leprosin A.

When the long-term-treated leprosy patients were separated into MB and PB groups, no considerable difference was found between them.

Individually comparison of those long-treated leprosy patients who

had received immunotherapy a year earlier with those who had received placebo, showed no statistically significant difference with any of the reagents.

The levels of $\%G_0$ are displayed in Figure 11. As seen in the same figure, 6/43 patients showed raised levels of G_0 (percentages of more than 10). Of the six raised levels of G_0, five had been found to be TB-PCR positive in the *M. tuberculosis* DNA study (Chapter 6). As there were only six TB-PCR positive patients in total, 5/6 (83.3%) were confirmed by the G_0 assay.

IgG antibody to the 65 kDa hsp did not correlate with any of the groups of leprosy patients according to type or PCR findings. However, it had a strong correlation with past immunotherapy (Table 14).

In the second sampling visit, serum samples were obtained particularly from PCR-positive patients. The serological results of these repeat sera was very similar to the results of the first series of serological tests, reconfirming the results.

7.3. Discussion

In leprosy, it has been shown that the decrease in antibody activity was most significant in the IgG assay (Brett *et al.*, 1983). IgG antibody levels therefore appear to be the most sensitive indicator especially

during the long-term treatment of leprosy.

In the present study, PGL-I ELISA proved to be a satisfactory technique for the detection of IgG antibody to *M. leprae*. In comparison with antibody levels detected to the crude antigens used in this study, very low levels of IgG antibody to the PGL-I antigen of *M. leprae* were found. A similar finding has been reported before, using glycolipid antigen of *M. leprae* compared with crude sonicates (Brett *et al.*, 1983). However, in this investigation of PGL-I ELISA no statistically significant differences were found between groups of healthy control persons, active TB patients, TB-PCR positive leprosy patients, and TB-PCR negative leprosy patients. On the other hand, statistically significant differences were found between groups of active LL and active TB patients, Lepr-PCR positive and TB-PCR positive leprosy patients, Lepr-PCR positive and Lepr-PCR negative leprosy patients. This suggests that PGL-I ELISA can be specifically used as a diagnostic method in serological studies of leprosy patients.

The high specificity of PGL-I ELISA obtained in this study confirms the effectiveness of this technique for the serodiagnosis of leprosy patients.

The finding of the greatest seropositivity for PGL-I in active LL and Lepr-PCR positive leprosy patients also supports the value of PGL-I ELISA in the serodiagnosis of leprosy patients.

The finding of more correlation between PGL-I seropositivity and

leprosin A-positivity but not with the three other skin test reagents (tuberculin, scrofulin, and vaccin) (Table 13), shows the usefulness of leprosin A skin-testing for leprosy.

The difference in PGL-I IgG between long-treated MB and PB patients was not significant. An explanation for this may be that the PB patients studied included more Lepr-PCR positive cases than were found among MB patients. In support of this, in another study (Lefford *et al.*, 1991) it was found that the PGL-I IgM ELISA may have its greatest diagnostic value in PB disease. In the same study, it was inferred that casual exposure to *M. leprae* or subclinical infection, is insufficient to induce a detectable humoral immune response to PGL-I. Further studies of antibodies to PGL-I on treated MB and PB patients are needed to establish the difference between these two groups.

From the data on mean values of different groups (Figures 7 and 8), taking the following steps might lead to a means of serodiagnosis for tuberculosis in leprosy patients.

1) In patients with mycobacterial disease, an increase of the mean value for son. tb above the mean value for son. lepr, enables calculation of the son. tb/son. lepr ratio.

2) Exclusively in active TB and TB-PCR positive groups the son. tb/son. lepr ratio exceeds 1.5 and reaches to high ratios of 1.6 and 1.8 respectively.

Considering the latter step, it appears that son. tb/son. lepr ratios of 1.5

and above indicate infection with *M. tuberculosis.* Further investigations are needed to confirm this and determine the usefulness of crude antigens of *M. tuberculosis* in the serodiagnosis of tuberculosis.

The finding of 83.3% elevated levels of G_0 in TB-PCR positive leprosy patients indicates the usefulness of G_0 assay in identifying tuberculosis in leprosy patients. Although the function of G_0 is not completely clear, the effectiveness of G_0 in diagnosis and its association with a small group of diseases has been proved (Parekh *et al.*, 1985; Rademacher *et al.*, 1988; Rook *et al.*, 1988; Rook *et al.*, 1989; Rook *et al.*, 1991b; Rook *et al.*, 1994). In addition to tuberculosis, high levels of G_0 are found in erythema nodosum leprosum (ENL), rheumathoid arthritis, Crohn's disease and schistosomiasis. In leprosy cases without ENL, levels of G_0 are usually within normal limits.

In conclusion, the comparative results of PGL-I ELISA work and *M. leprae* DNA study demonstrated that PGL-I ELISA can differentiate between leprosy patients positive or negative by PCR. Thus it could be applied in relapse or re-infection studies of leprosy. A ratio of antibodies to antigens of tuberculosis and leprosy, and G_0 were found to be potentially useful serological markers for tuberculosis in leprosy patients. Antibodies to the 65 kDa hsp showed a potentially important relationship to past immunotherapy which is discussed in Chapter 9.

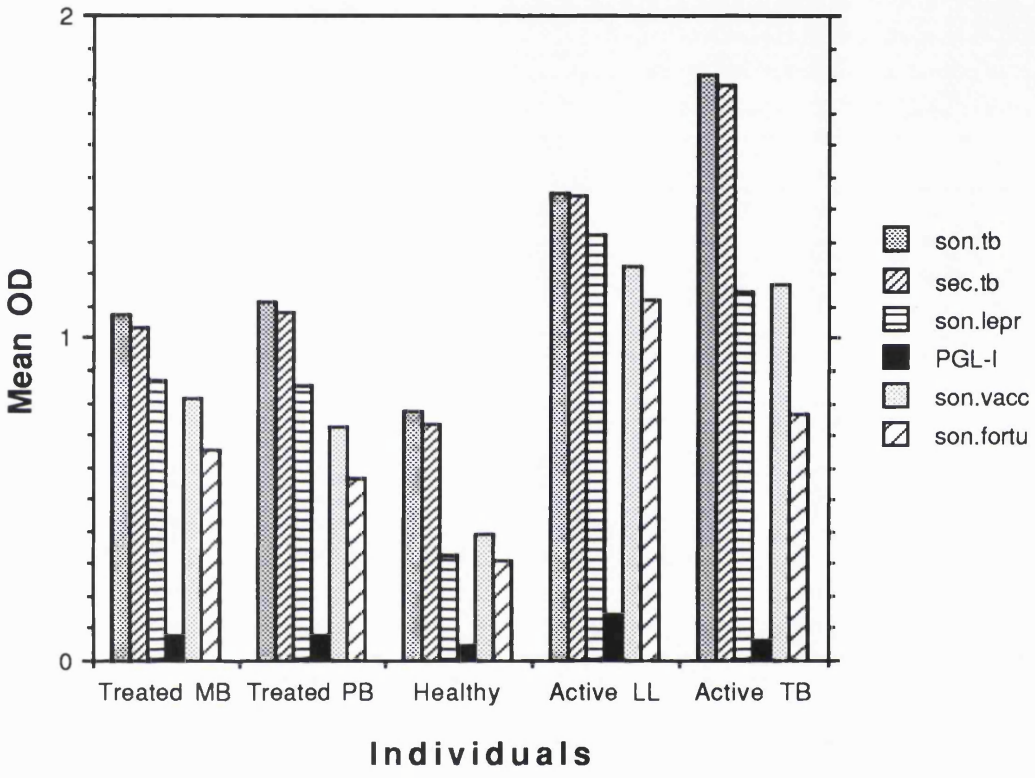


Figure 7. IgG response to mycobacterial antigens in MB and PB patients and control groups.

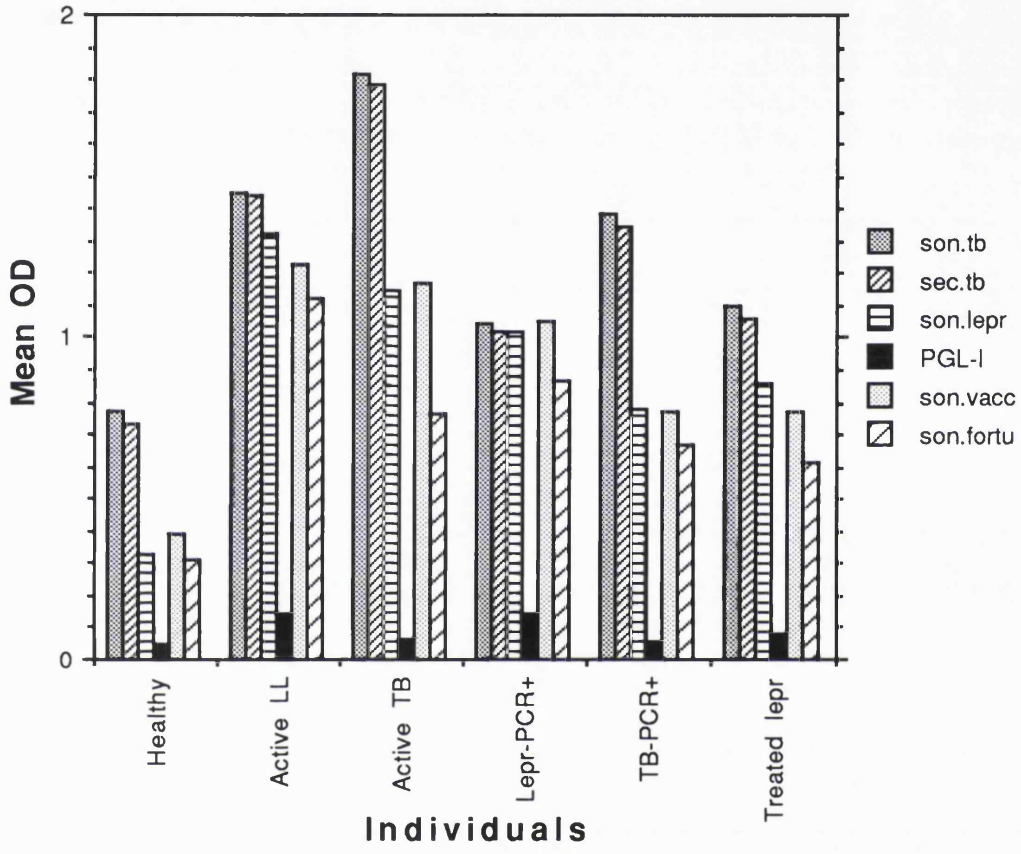


Figure 8. IgG response to mycobacterial antigens in control groups, PCR-positive patients and treated leprosy patients.

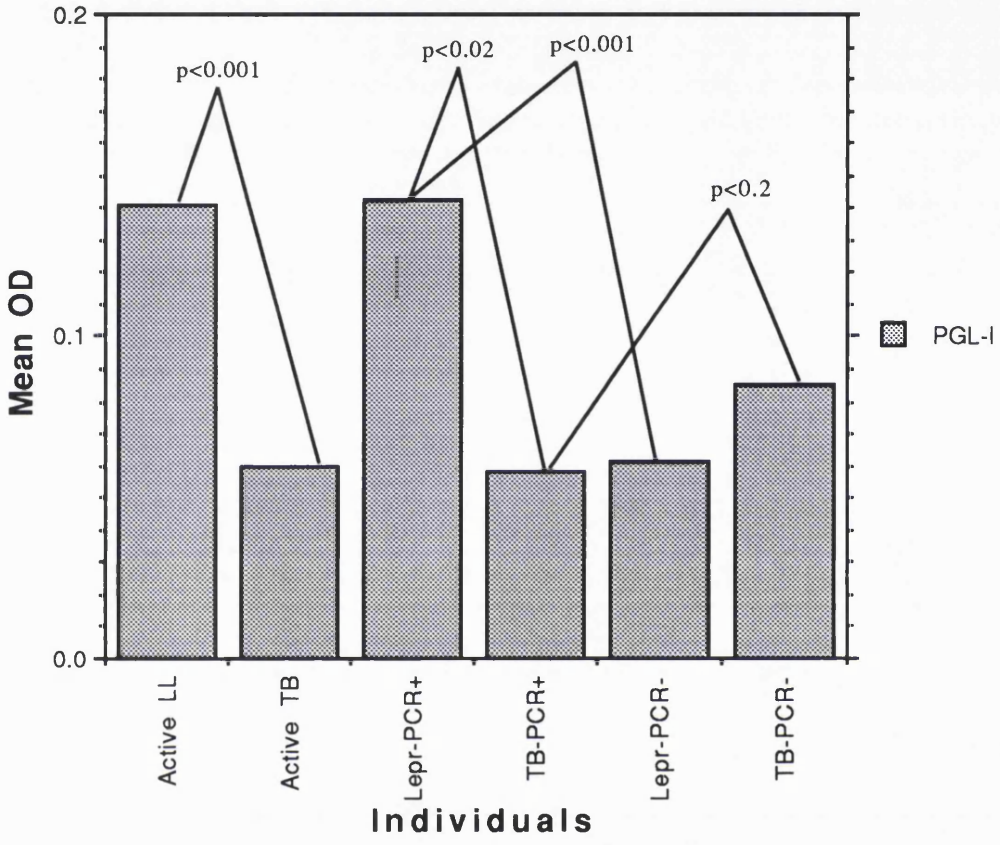


Figure 9. IgG to PGL-I in six different groups.

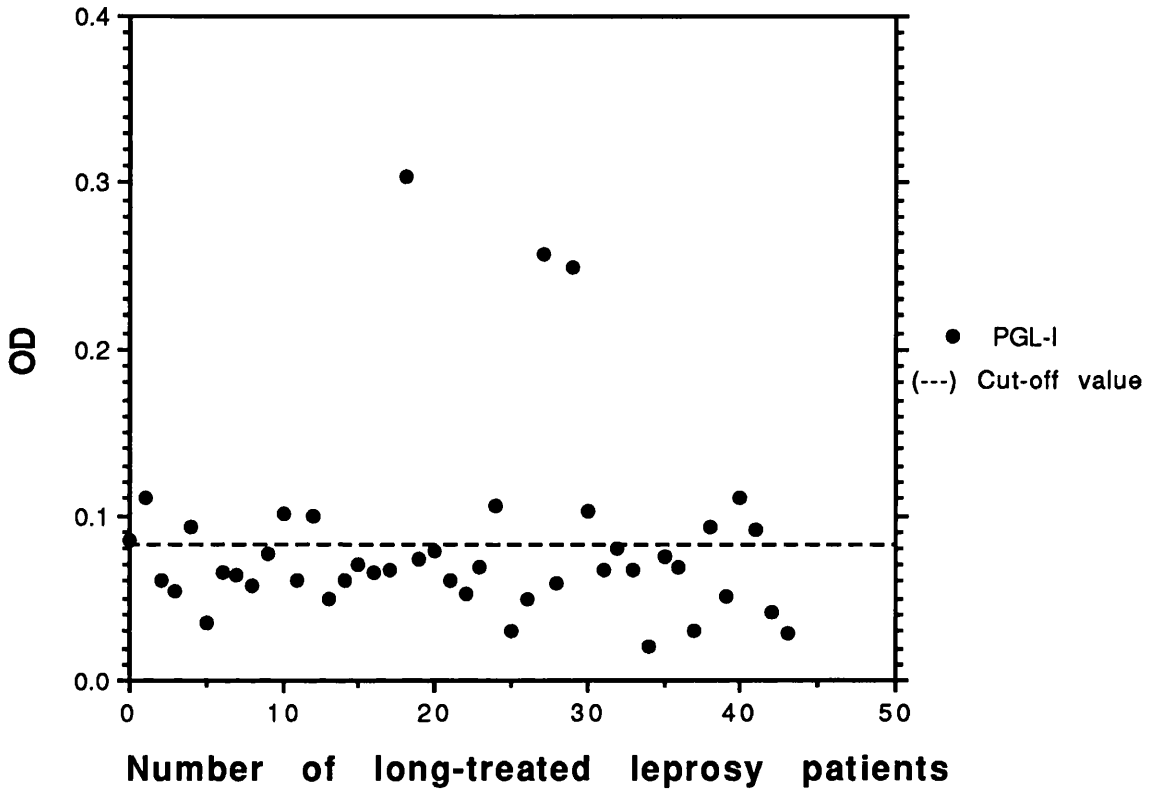


Figure 10. OD results with PGL-I for long-treated leprosy serum group.

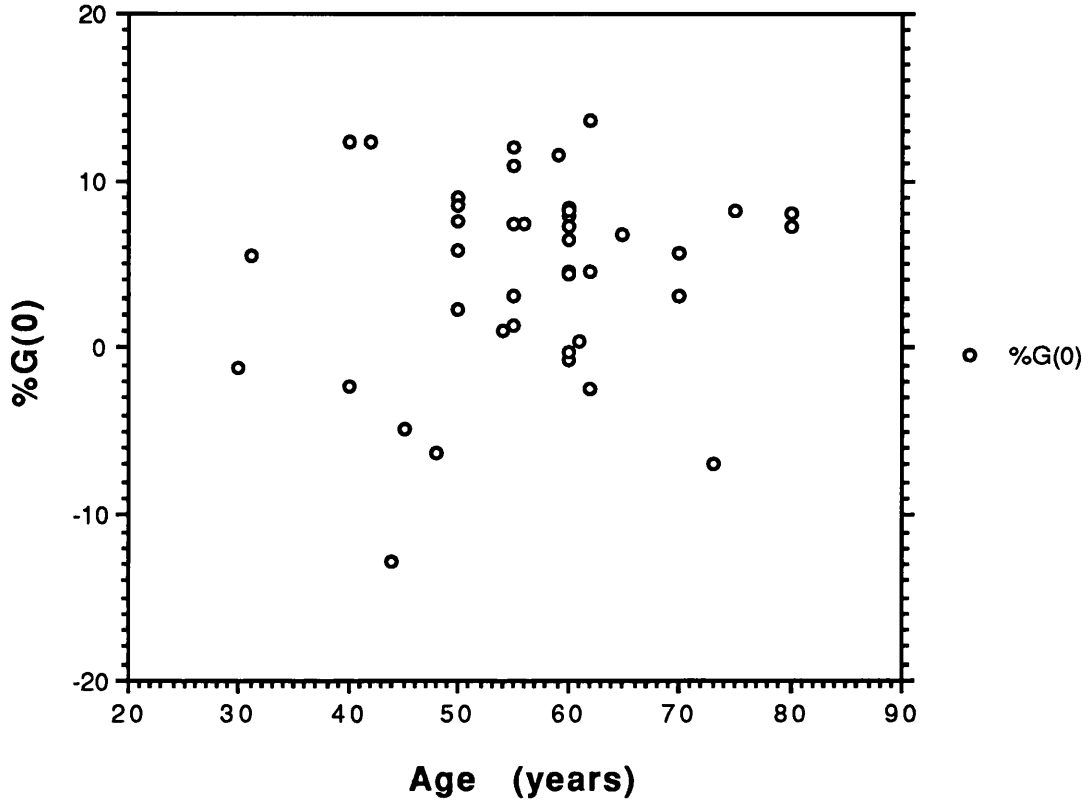


Figure 11. The levels of $\%G_0$ in long-treated leprosy patients.

Table 13. Correlation between seropositivity for PGL-I and skin test positivity with 4 new tuberculins.

Skin-testing	No. of skin test positive* patients	No. (%) of PGL-I seropositive patients amongst the skin test positive group (tested a year later)
Tuberculin	39	8 (20.5%)
Leprosin A	13	7 (53.8%)
Scrofulin	15	4 (26.7%)
Vaccin	14	6 (42.9%)

*Reactions of 2 mm or more are taken as positive responses with these reagents(Stanford *et al.*, 1989; Ghazi Saidi *et al.*, 1989).

Table 14. IgG titres to 65 kDa hsp in *M. vaccae* recipients and control groups of long-term leprosy patients.

Receiving IT	No. of patients	Mean IgG titre (± SD) to hsp (tested a year later)
M. vaccae (yes)	22	0.170 ± 0.136
		*
Saline (no)	21	0.313 ± 0.225

*$p < 0.01$

CHAPTER 8

Additional Investigations

In addition to the chief work for my PhD, I had the opportunity to do two more sets of investigations on tuberculosis and leprosy which were more or less related to my basic project. These two are explained independently in this chapter.

(I)

Evaluation of Conventional Bacteriological Methods and PCR for the Diagnosis of Tuberculous Meningitis

8 (I).1. Introduction

The most dangerous form of extrapulmonary tuberculosis is tuberculous meningitis (TBM) which occurs in 7-12% of tuberculous patients in developing countries (Tandon, 1978). TBM can occur at any age except in the new born. Patients with TBM always have a focus of infection elsewhere, but, one in four have no clinical or historical evidence of such an infection. Occasionally, the onset is much more rapid and may be mistaken for a sub-arachnoid-haemorrhage (Braude, 1981). In spite of the availability of effective chemotherapy, the mortality and morbidity of TBM remain high. Delay in the diagnosis of

TBM is directly related to poor outcome; there are neurological sequelae in 20-25% of patients who do not receive early treatment (Molavi and LeFrock, 1985).

As referred to in Chapter 2, the diagnosis and confirmation of tubercle bacilli in specimens by conventional bacteriological methods usually requires several weeks. Therefore, new and more rapid techniques are needed to solve this problem. Recently, amplification of mycobacterial DNA sequences by the polymerase chain raction (PCR) has shown promise as a rapid and sensitive method for detection of mycobacteria in clinical specimens.

In this investigation, polymerase chain reaction (PCR) was performed to detect specific insertion sequences (IS*6110*) of *M. tuberculosis* DNA (as described previously in Chapters 4 and 6) in cerebrospinal fluid (CSF) specimens from patients suspected to have TBM.

The purpose of the present study was to investigate whether PCR detects tubercle bacilli in CSF specimens that are missed by direct microscopy and culture, and if so, whether PCR has a better diagnostic value than conventional methods. In fact, in view of the importance of the early diagnosis of TBM, this study compares the efficiency of the PCR with conventional methods (acid-fast microscopy and culture) for the detection of *M. tuberculosis* in CSF specimens from patients suspected of having TBM.

8 (I).2. Patients and clinical specimens

CSF specimens from 29 patients thought to have TBM, received at two different hospitals in Tabriz, Iran during 1993 were examined in the laboratory by direct microscopy and culture. Aliquots of the CSF specimens were brought to London for PCR examination. CSF specimens from the 6 non-tuberculous patients as a control group, were also examined by all the three above mentioned methods.

8 (I).3. Materials and methods

Acid-fast microscopy (AFM). Fixed smears of CSF prepared from centrifuged sediments were stained by the Ziehl-Neelsen (ZN) method for acid fast bacilli (AFB) as described in Chapter 4. After staining, more than 20 fields of each smear were examined carefully under the light microscope using the oil immersion (X 100) lens.

Culture. The sediments of centrifuged CSF specimens were inoculated onto Lowenstein-Jensen (LJ) medium without delay, and incubated at 37°C for 6-8 weeks. The slopes were examined weekly. Positive

cultures were examined by selected tests as mentioned in Chapter 2 (Table 3) for the definitive identification of *Mycobacterium tuberculosis.*

DNA extraction from clinical specimens and preparation of chromosomal DNA. The GuSCN-diatoms method was applied for DNA extraction as described in Chapter 4 and discussed in Chapter 5 (using 50 µl of the sediment of each spun CSF). The *M. tuberculosis* chromosomal DNA preparation used was the same as described in Chapter 4.

Selection of primers and PCR procedure. The primers used for the specific amplification of *M. tuberculosis* DNA were the IS*6110* primers as explained in Chapters 4 and 6, with the same PCR procedure.

8 (I).4. Results

CSF specimens obtained from 29 patients suspected of TBM were examined by direct microscopy for AFB, culture, and PCR. No PCR inhibitors were found in the PCR negative specimens. The results are summarised in Table 15. Of the 29 CSF specimens, only one was

positive by AFM (3.4%), and five were culture positive (17.2%), whilst 25 specimens were found to be PCR positive for *M. tuberculosis* (86.2%).

The results of AFM, culture and PCR were negative in CSF specimens from the 6 non-tuberculous patients (control group).

Table 16 shows that 20 cases were positive by PCR alone (69%), whereas 4 cases were positive by both culture and PCR (13.8%) and only one case was positive by all three, AFM, culture, and PCR (3.4%). No positive results were obtained by AFM or by culture alone.

The results of this investigation demonstrated that the positivity rate of PCR was 5 times higher than the positivity rate of culture, and 25 times higher than the positivity rate of direct microscopy in TBM patients.

8 (I).5. Discussion

This study shows that the efficiency of PCR is considerably higher than direct microscopy and culture for the early diagnosis of TBM (Table 15). PCR also has the advantage that results are rapidly available allowing diagnosis to be made earlier. The accuracy of this study is demonstrated by the fact that the control group gave negative results.

Conventional bacteriological methods such as direct microscopy and

culture are not sufficient for the early diagnosis of TBM because there are too few bacilli in the cerebrospinal fluid (CSF) to be demonstrated by direct microscopy and on the other hand, successful cultural identification of tubercle bacilli takes about 7 weeks. It seems that by the development of the polymerase chain reaction, one of the most sensitive DNA-based assays, it is now possible to overcome these shortcomings.

The high sensitivity of this PCR and its detection limit (fewer than 10 bacilli) has been discussed in Chapter 6. It is believed that such a detection limit of this PCR combined with the application of the previously discussed GuSCN-diatoms method of DNA extraction (Chapter 5) from clinical specimens can provide a powerful tool for the specific and rapid diagnosis of paucibacillary cases of tuberculosis.

In this study, the 86.2% positivity rate of PCR in a small selection of patients suspected of TBM, is remarkable. In view of the fact that there are too few bacilli in CSF specimens from TBM patients to be demonstrated by direct microscopy, and on the other hand cultural examination of CSF specimen from TBM patient takes several weeks, it is desirable to carry out PCR as highly efficient technique, for the rapid diagnosis of TBM, even though conventional diagnostic methods are much cheaper. PCR can specifically identify *M. tuberculosis* in a clinical specimen within 7-8 hours. The use of PCR for the identification of other mycobacteria has recently been published

(Telenti *et al.*, 1993) and this, when developed further, might enable reference TB laboratories to identify all species of mycobacteria in a matter of hours rather than months.

The results of this study also indicate that PCR can be used alone as a reliable test for the diagnosis of TBM, considering that 20 CSF specimens out of 29 (69%) were positive by only PCR (Table 16).

Another advantage of PCR is that it may detect DNA of *M. tuberculosis* after chemotherapy has started whereas culture usually fails under these conditions. Because of the dangers of TBM, treatment is often started even before the patient reaches hospital and diagnostic specimens are taken.

In conclusion, the results of this study suggest that in view of the specificity, sensitivity, and rapidity of this PCR, it can be applied as a powerful technique for the diagnosis of difficult cases of tuberculosis such as TBM when a fast diagnosis is essential.

Table 15. Evaluation of PCR in CSF specimens from patients suspected of tuberculous meningitis (TBM).

Specimen	No. tested	Positive by:		
		AFM *	Culture	PCR **
CSF from highly probable TBM patients	29	1 (3.4%)	5 (17.2%)	25 (86.2%)
CSF from non-tuberculous patients (control group)	6	0	0	0

*AFM, acid fast microscopy
**PCR, polymerase chain reaction

Table 16. The results of patients suspected of TBM in detail.

Total No. of patients suspected of TBM	No. (%) of TBM patients with positive results by:				
	only AFM	only culture	only PCR	both culture & PCR	all three AFM, culture & PCR
29	0 (0%)	0 (0%)	20 (69%)	4 (13.8%)	1 (3.4%)

(II)

Presence of *M. leprae* DNA in Ancient Bone

8 (II).1. Introduction

It has been shown that leprosy leaves a typical series of bony changes recognizable in human skeletons (Brothwell, 1981), although sometimes these may be confused with those resulting from other diseases (Ortner and Putschar, 1981). Such bones have been recovered from burials thousands of years old, amongst the oldest being a specimen from the Hellenistic Period (200 BC) in Egypt (Dzierzykray-Rogalski, 1980). A ceramic jar with the face of a man who looks as if he may have had the disease is reported from Tel Beit Shean on the banks of River Jordan dated 1400-1300 BC (Ortner and Putschar, 1981).

Human DNA degrades rapidly after death. Paabo (1993) found the average length of DNA to be around 100 base pairs in specimens varying in age from 4000 to 13000 years after death. None the less, the very resistant cell walls of mycobacteria may provide an effective barrier protecting the bacterial DNA until after the DNAses released during decomposition have become inactivated.

In a study performed in this laboratory by Eshetu Lemma, DNA of 123 base pairs was detected by using the IS*6110* primers specific for an

insertion sequence believed to be limited to *M. tuberculosis*, in bones about 1000 years old (Spigelman and Lemma, 1993).

Following my success in detecting genomic DNA of *Mycobacterium leprae* by PCR in the tissues of long-treated leprosy patients in whom bacilli could not be found by microscopy, I applied the same system to archaeological specimens.

8 (II).2. Samples

Very small pieces of five ancient bone samples were generously supplied and brought by Dr M. Spigelman of the Institute of Archaeology, UCL. These were phalanges (11-13th century AD), rib (11-13th century AD), talus (mediaeval), metatarsal (600 AD), rib (300 AD) and originally were from Cambridge, Cambridge, Ancient Monuments, Jerusalem, and Romano-British respectively. Other than two rib samples which were used as controls, the three other bone samples were morphologically suspected of leprosy.

8 (II).3. Materials and methods

DNA extraction from bone samples and preparation of

chromosomal DNA. Each bone sample was individually, carefully and thoroughly ground in a sterile laboratory mortar with a sterile pestle using a safety cabinet. A few mg of each sample was prepared by the GuSCN-diatoms method of DNA extraction as explained in Chapter 4. The *M. leprae* chromosomal DNA preparation used as a positive control in the PCR assay was the same as explained in Chapter 4.

Selection of primers and PCR procedure. Two sets of primers were used. A set of primers used for the specific amplification of *M. leprae* DNA (species-specific primers) were S13 and S62 of Hartskeerl *et al.* (1989) as described in Chapters 4 and 5. The genus-specific primers used were Tb11 and Tb12 of Telenti *et al.* (1993) which amplify nucleotide sequences of 439-bp encoding part of the 65 kDa protein of all mycobacteria. The sequence of the latter set of primers (synthesized by Oswel DNA Service, Edinburgh, UK) were:

Tb11 (5'-ACCAACGATGGTGTGTCCAT-3') and

Tb12 (5'-CTTGTCGAACCGCATACCCT-3')

The PCR procedures used were the same as described in Chapter 4.

8 (II).4. Results and discussion

The results are displayed in Table 17. Sample 4, seen in Figure 12 with

a normal metatarsal on the left for comparison, had the best morphological evidence of clinical leprosy of all those studied. It was in sample 4 that a very weak band of mycobacterial DNA was detected by both sets of primers, one of which was specific for *M. leprae.*

A ^{14}C date for the site, based on wood found in the grave, is 600 AD ±50 years. The grave is from a site where there was a massacre of Christians by the Persians in 614 AD (Gill, 1992), in the grounds of the Monastery of Saint John the Baptist, on the River Jordan at the spot where it is believed that John the Baptist baptized Jesus. This is also the traditional site for the ceremony of the washing of the 'leper' in Christian sources.

The PCR product from the gene encoding the 65 kDa protein primers (Telenti *et al.*, 1993) can be subjected to restriction enzyme analysis, enabling differentiation to the species or subspecies level. Restriction patterns were not described by Telenti *et al.* (1993) for *M. leprae*, although preliminary work on sample 4 has indicated that there are two fragments of 125 and 325 base pairs, produced by digestion with *Bst*EII enzyme and two fragments of 128 and 260 base pairs produced by *Hae*III enzyme digestion (Rafi *et al.*, 1994). However, at present, insufficient PCR product from the Lepr-PCR positive ancient bone (sample 4) has been produced to confirm whether or not the fragments from modern *M. leprae* and ancient bone are identical (Rafi *et al.*, 1994).

Sample 1, also thought to come from a patient with leprosy, was PCR-positive by the genus-specific primers alone, and possibly analysis of restriction fragments will reveal more details about this sample.

Sample 3 had been used previously to search for DNA from *M. tuberculosis* and this was detected (Spigelman and Lemma, 1993), despite the morphological appearance suggesting that this was a case of leprosy. The length of DNA amplified in the tuberculosis study was only 123 base pairs, and according to Paabo (1993) a piece of DNA of this length might be expected to have survived. It proved negative with PCR using the genus-specific primers, however, but this could be explained by the greater length of intact DNA (439-bp) required for this PCR. Both Paabo (1993) and Hagelberg (1989) consider that the optimum size of surviving ancient DNA chains is no more than 200 base pairs. This may well be true for DNA chains of human DNA but it is felt that the DNA of mycobacteria in particular, because of the extra protection provided by the thick cell wall, may well be capable of yielding longer chained segments of DNA.

Co-infection of leprosy patients with tubercle bacilli was a frequent occurrence in the days before antibiotics. Indeed, in the past, tuberculosis was a common cause of death for patients afflicted with leprosy (Harrison *et al.*, 1962). The use of primers selecting a shorter length of DNA specific for *M. leprae* might confirm this situation in sample 3. In addition, restriction analysis of the PCR product available

from sample 1 may show that the infecting organism was really the tubercle bacillus.

It is believed that the results of this investigation are important to the study of the history of medicine, and may help to establish molecular palaeobacteriology as a new scientific approach to an old field. The ability to amplify genetic material from ancient bacteria, and then sequence the results, could have significant implications medically. It might in fact be possible to note some genetic changes, which may help identify variations in virulence of certain organisms. Changes in bacterial DNA may be assessed and perhaps used as a dating technique by comparing them with modern organisms of the same species. The theory of the pre-existence of antibiotic resistance can also be explored.

Ancient DNA from *Escherichia coli* has also been isolated from the bowel contents of Lindow Man, an Iron age (500 BC) bog body (Fricker *et al.*, in preparation), thus proving that organisms other than from the well-protected mycobacteria can survive from ancient times.

The significance of this study and similar rare studies is that there is no need to be reliant on recognizable morphological changes in bone for the diagnosis of bacterial infection. It is possible that any bacterial infection at time of death may now be potentially diagnosable provided the correct primers are used. A note of caution must, however, be cast here, as there is still no familiarity with the conditions that allow DNA preservation. Primers are specific for a short sequence of DNA, the

absence of this sequence does not necessarily signify that other segments of DNA from the organism are also absent, thus a negative result does not mean that a particular organism was not present in the specimen or that all the DNA chains of that organism are absent.

Perhaps these techniques will provide the answers to McNeill's (1976) statement 'proof one way or another, awaits development of precise and reliable methods whereby organisms causing lesions in ancient bones can be recognised'.

Table 17. Ancient bone samples characteristics and PCR results.

No.	Sample (origin)	Period	Morphological diagnosis	*M. leprae* specific PCR	Mycobacteria specific PCR
1	Phalanges (Cambridge)	11-13th century AD	Leprosy	-	+
2	Rib (Cambridge)	11-13th century AD	Control	-	-
3	Talus (Ancient Monuments)	Mediaeval	Leprosy	-	-
4	Metatarsal (Jerusalem)	600 AD	Leprosy	+	+
5	Rib (Romano-British)	300 AD	Control	-	-

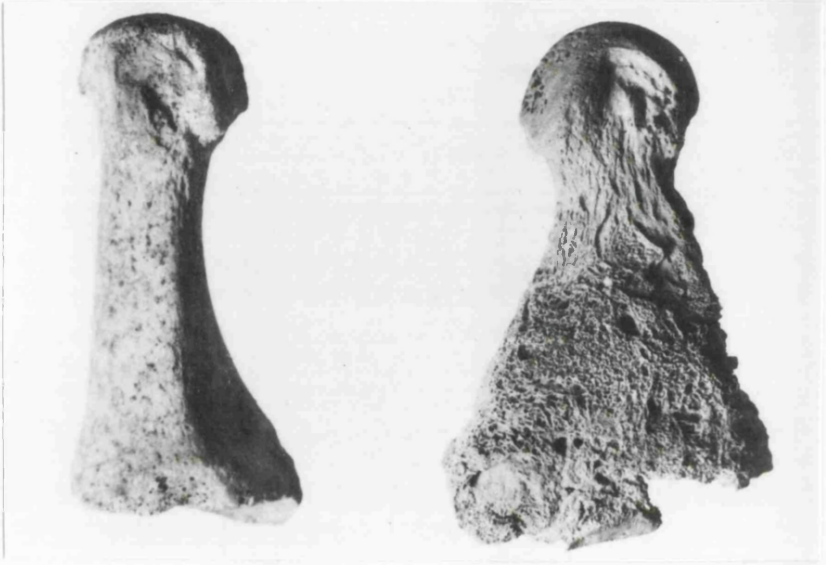


Figure 12. 1st metatarsal of a leprosy patient, with a normal control on left.

CHAPTER 9

General Discussion

The main objective of the study described in this thesis was the development and subsequent application of the PCR assay for the identification of *M. leprae* and *M. tuberculosis* and confirmation of these organisms by the serological tests in specimens from selected long-term-treated leprosy patients of the Baba Baghi Leprosy Sanatorium. In parallel with the main project, the results obtained were compared with skin test results of the preceding visit led by Dr J.L. Stanford a year earlier, at which time immunotherapy with *M. vaccae* had been given to about half of the patients.

Throughout, the study showed that PCR was a powerful, practical, rapid and sensitive procedure which had potential uses for the specific diagnosis of leprosy and tuberculosis in both research and the clinical laboratory. Of special interest, it could be used to detect apparently silent infections of leprosy patients with tubercle bacilli. In leprosy research, this technique offers the possibility of detecting persistent bacilli, and enabling detection of relapse or re-infection amongst long-term-treated leprosy patients in a leprosarium or an endemic area. My study shows that the PCR technique will be useful in follow-up examinations of fully treated patients leading to better control of

disease. Consequently the progress and result of anti-bacterial drug-therapy for leprosy can be revealed by the technique.

The diagnosis of *M. tuberculosis* infection in leprosy patients is also possible by the application of the *M. tuberculosis*-specific PCR-based diagnostic test (using IS*6110* primers) and thus the association between leprosy and tuberculosis can be investigated more effectively than before.

The possibility of the identification of *M. leprae* in ancient materials is offered by the use of the *M. leprae*-specific PCR-based detection technique (using S13 and S62 primers). The finding of ancient DNA of *M. leprae* described in this thesis and the isolation of ancient *M. tuberculosis* DNA reported by two other studies (Spigelman and Lemma, 1993; Salo *et al.*, 1994), promise the establishment of molecular palaeobacteriology as a new scientific field. These studies may also help to understand the evolution of diseases, which could have important implications for their cure.

In tuberculosis research, using the *M. tuberculosis*-specific PCR-based assay (with IS*6110* primers), the possibility will exist to diagnose paucibacillary situations of tuberculosis such as TBM. A recent study (Hawkey PM, 1995) suggests that traditional techniques (direct microscopy and culture) are still needed until the variables of extraction techniques, sensitivity and specificity, particularly in relation to dead mycobacteria, are better understood.

Nowadays, using techniques based on PCR, in addition to the rapid diagnosis of leprosy and tuberculosis in clinical specimens that are negative by microscopical examination, it is possible (Grosset and Mouton, 1995) to:

1) Rapidly detect whether acid-fast bacilli identified by microscopical examination in clinical samples are *M. leprae/M. tuberculosis* or atypical mycobacteria.

2) Detect whether or not identified *M. leprae* and *M. tuberculosis* in different specimens have a common origin in the context of epidemiological studies.

3) Detect the presence of genetic modifications known to be associated with resistance to some anti-mycobacterial agents.

Evaluation of the different DNA extraction methods proved that the GuSCN-diatoms procedure could be a simple and practical method for routine use in the preparation of clinical samples for PCR assays. Considering that this method was much cheaper, it could therefore be readily established in any reference leprosy and tuberculosis laboratory in developing countries. As explained in Chapter 5, in view of the fact that the GuSCN-diatoms technique is effective with *M. leprae*, which has a very refractory cell wall, it is believed that this technique will be of value for the DNA extraction of other bacteria.

As the skin test (done a year earlier by Dr J.L. Stanford and colleagues) results of Chapters 5 and 6 demonstrate, the finding of

more correlation between PCR-positivity of leprosy and leprosin A-positivity in comparison with the positivity of three other reagents (T, S, and V), indicates that leprosin A (LA) skin-testing is a useful epidemiological guide to the extent of exposure of a community to the leprosy bacillus where the use of PCR is impossible. However, skin-testing is of no direct diagnostic value in clinical leprosy (Grange, 1988). One study (Stanford *et al.*, 1980) appeared to show that skin-testing on leprosy patients was of value as a measure of protection from infection, but whether even this will prove useful at the individual level is far from certain. In common with the tuberculin reaction, a positive response to LA indicates previous exposure to the relevant antigens or cross reactive response to common antigens. Thus the test may be positive in healthy contacts and non-contacts as well as in immunologically reactive patients (Grange, 1988). The usefulness of LA skin-testing for leprosy was confirmed when the leprosy serological results (Chapter 7) showed more relation with LA-positivity.

Correlations between the results of testing with LA and other test reagents can be made. Of the 14 patients producing positive responses to vaccin (V), eight produced positive responses to LA (57.1%) and of those negative to V, five were positive to LA (5/30, 16.7%). Of the 15 patients producing positive responses to scrofulin (S), seven were LA-positive (46.7%) and of those negative to S, six were positive to LA

(6/29, 20.7%). Of the 39 tuberculin-positive patients, nine were LA-positive (23.1%) and of those negative to T, only one was positive to LA (1/5, 20%). The nature of the relationships reflected by correlations of skin test results is not clear and again simple cross-reactivity does not appear to be the explanation (Stanford *et al.*, 1980).

The finding of a close correlation between PCR-positivity of tuberculosis and tuberculin-positivity confirmed the importance of tuberculin testing as a marker of *M. tuberculosis* infection in long-treated leprosy patients. All the six TB-PCR positive patients of the study had previously shown strong responses to tuberculin (T) (defined as more than 12 mm of induration), but no responses or a weak one to leprosin A (LA), scrofulin (S), and vaccin (V). A positive tuberculin test indicates that the patient has experienced immunologically effective contact with mycobacteria. This may be due to active tuberculosis, past infection, past BCG vaccination or sensitization by environmental mycobacteria. Reactions due to environmental mycobacteria can usually be differentiated from those due to *M. tuberculosis* infection or BCG vaccination on the basis of size (Grange, 1988). In a recent study (Dy and Victorio-Navarra, 1994), the enhanced diagnostic use of the tuberculin skin test in recognizing extrapulmonary tuberculosis with or without pulmonary involvement, has been confirmed.

Correlations between the results of testing with T and other reagents can be determined. Of the 14 patients producing positive responses to

V, all were T-positive (100%) and of those negative to V, 25 were positive to T (25/30, 83.3%). Of the 15 patients producing positive responses to S, all produced positive responses to T (100%) and of those negative to S, 24 were positive to T (24/29, 82.8%). Of the 13 LA-positive patients, 12 patients produced positive responses to T (92.3%) and of those negative to LA, 27 were positive to T (27/31, 87.1%). However, as mentioned earlier, the nature of this kind of correlation and its reflected relationships is not clear and simple cross-reactivity does not seem to explain the correlation.

The important finding, of more TB-PCR positive results from patients who had previously shown Koch responses to tuberculin than from those who had previously shown non-Koch responses to tuberculin (5/21 compared with 1/23; p=0.074), reconfirms the usefulness of tuberculin testing amongst long-treated leprosy patients in order to ascertain Koch-type responses, and to indicate any *M. tuberculosis* infections among such patients. Because skin-testing studies are much cheaper than PCR investigations, it would be wise to do skin-testing prior to any PCR on such patients. It is evident that PCR can confirm the results of skin-testing, and identify the suspected tuberculosis cases in a leprosarium.

No difference in PCR-positivity of tuberculosis was found between two groups of long-treated leprosy patients who had received immunotherapy or a placebo a year earlier. It appears it is not possible

to judge the effectiveness of the immunotherapy of tuberculosis, where the patients studied are leprosy patients and diagnosed TB-PCR positive leprosy patients were small in number.

The comparative results of the leprosy study described in this thesis indicate that although the serological tests are not as sensitive as the PCR-based assays, serological methods can be used as an affordable alternative to conventional diagnostic methods where it is difficult to find out leprosy bacilli in clinical specimens microscopically. Serological techniques may also be applied as confirmatory tests to PCR-based detection methods in leprosy studies. Thus serological techniques can be used in relapse, re-infection, and treatment follow-up studies of "fully treated" long-term leprosy. Leprosy bacilli contain antigenic components cross-reacting with several other mycobacteria. These cross-reacting components, both from *M. leprae* and from other mycobacteria, have been and are still used to detect and quantitate the activity of anti-mycobacterial antibodies in serum samples from leprosy patients (Melsom, 1983).

The results from the cross-sectional studies suggest that PGL-I antibodies provide a sensitive test for leprosy (Smith, 1992). In support of this, the serological investigation described in Chapter 7 of this thesis confirmed that PGL-I, a highly specific antigen of *M. leprae* could be used in IgG ELISA to detect the antibody titre to this antigen in the serodiagnosis of leprosy. It is thought that when antibodies are

detected, this indicates a present or past infection with *M. leprae*, whether or not there are clinical signs. However, there is a problem; as shown in Figure 10 of Chapter 7, these seem to be two populations of serological responders to PGL-I, and the only way to obtain high sensitivity is to use a cut-off level which bisects one of the populations rather than separating between them. Thus it seems unlikely that the test has real value.

Serological examinations are likely to be more useful for diagnosis when they are considered together with other diagnostic tests such as PCR assays. If detection of species-specific antibodies indicated infection with *M. leprae*, serological assays would greatly advance the understanding of the epidemiology of the disease.

Detection of agalactosyl IgG (G_0) has proved useful in the patients I examined. Unlike tuberculosis, G_0 is only raised in early ENL in leprosy, thus in the long-term-treated patients I studied, it acted as a sentinel of silent tuberculosis, correlating with a positive TB-PCR on sputum samples. Much easier than the TB-PCR, the G_0 assay might be used to screen long-term-treated leprosy patients for those infected with tubercle bacilli, and suitable treatment might be implemented.

Although serology has generally not been very useful in the diagnosis of tuberculosis, combined infection with *M. leprae* may be a different matter. Thus the simple ratio between antibody levels to *M. tuberculosis* sonicate and *M. leprae* sonicate may offer a useful

diagnostic procedure.

The interesting finding that more Lepr-PCR positive patients were found among patients who had received saline as placebo one year earlier than amongst those who had received *M. vaccae* as immunotherapy at the same time, was statistically significant ($p < 0.01$). It seems that this important finding may be helpful evidence for the effectiveness and value of the immunotherapy of leprosy with killed *M. vaccae*. It supports previous studies that have shown faster removal of microscopically visible leprosy bacilli after immunotherapy with *M. vaccae* in new patients with lepromatous leprosy receiving multi-drug therapy. In immunotherapy, the immunotherapeutic agent stimulates the patient's immune reactivity to treat the disease. *M. vaccae* as a powerful source of common mycobacterial antigen reintroduces recognition of the shared or species-specific antigens of the leprosy bacillus so that an effective immune response can be directed towards the leprosy bacillus (Stanford *et al.*, 1988); *M. vaccae* probably regulates the maturation of the various types of helper T-cells, and possibly it suppresses Th-2 mediated responses and enhances Th-1 mediated responses by blocking the influence of TNF (Stanford *et al.*, 1993). Recently, some of the same group of leprosy patients in the blinded randomized study at Baba Baghi leprosarium from whom my study patients were selected, have been examined by Dr N. Abbot for fingertip blood flow 18 months after *M. vaccae* or

placebo was given (Stanford, 1994). His results show that immunotherapy patients have fingers an average of 1°C warmer than those of placebo recipients, and also have significantly increased blood flow to their fingertips, as assessed by laser Doppler flowmetry. My finding of lowered IgG antibodies to the 65 kDa heat shock protein of *M. tuberculosis* in immunotherapy recipients fits in very well with Dr Abbot's results (Stanford, 1994). Recent work at the Institute of Biomedical Aging Research in Austria associates raised levels of antibody to the 65 kDa hsp to arteriosclerosis, presumably reflecting an underlying vasculitis (Wick *et al.*, 1995).

Ophthalmological examination of the patients at Baba Baghi by Professor Shams (Stanford, 1994) contemporaneous with Dr Abbot's investigation, showed a decrease in the numbers of cases of anterior uveitis in *M. vaccae* recipients. The combination of these observations suggests that immunotherapy with *M. vaccae* alleviates chronic inflammatory processes as well as leading to removal of leprosy bacilli. Together these powerfully support the potential value of immunotherapy in the treatment of leprosy, both as an infection and as a chronic disease.

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