IMMUNOGENICITY OF PLASMA MEMBRANE PROTEINS OF MYCOBACTERIAL SPECIES

A THESIS

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By

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Gratis.

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **IMMUNOGENICITY OF PLASMA MEMBRANE PROTEINS OF MYCOBACTERIAL SPECIES** in fulfilment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Biosciences and Biotechnology** of the University is an authentic record of my own work carried out during a period from **July' 1995 to August '1998** under the supervision of **Dr. A.K. RASTOGI** and **Dr. R.P. SINGH**.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University.

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ABSTRACT

Successive failures of integral vaccines in the prevention of mycobacterial diseases - tuberculosis and leprosy have intensified the quest for development of subunit vaccines resulting in exquisite definition of an array of mycobacterial antigens. The focus has obviously been on protein antigens as they are capable of generating a 'cell (T cell) mediated immunity' which is regarded as the principle host-protective mechanism against intracellular pathogens. Mycobacterial proteins well characterised to date are mainly the ones derived from cytosol, cell wall, or those released in the medium. Little knowledge is available regarding antigens localised within the mycobacterial plasma membrane. Yet, data from other intracellular pathogens, like Treponema pallidum, Leishmania spec., and Plasmodium falciparum, suggest that such proteins specially the detergent soluble 'integral membrane proteins' are highly immunogenic and in some cases protective.

This study is aimed at antigenic definition of plasma membrane proteins of BCG vaccine and identification of immunodominant T cell activating subunits. BCG despite all controversies is the only vaccine which has afforded considerable protection against tuberculosis and more so against leprosy. Apart from being clinically safe it is also bestowed with adjuvant properties. An understanding of immunodominant antigens of BCG is likely to provide clues to the development of more effective immunoprophylactic / immunotherapeutic agents based on cross-reactive

mycobacterial antigens.

Plasma membrane isolated from culture-grown BCG (Indian vaccine strain) was subjected to Triton X-114 based biphasic extraction procedure for isolation of 'peripheral' (water soluble) and 'integral'(detergent soluble) proteins (PMP and IMP). Distinction between the two protein pools was evident from results of SDS-PAGE and immunoblotting using antisera raised in rabbits. ELISA with a panel of WHO- IMMYC monoclonal antibodies against various mycobacterial antigens revealed that three well known antigens - 19kDa, 33/36kDa ("proline rich") and 38kDa (Pst-S homolog) were a part of IMP pool; and another such antigen, 14/16kDa α-crystalline homolg, partly constituted the PMP pool.

Apparently, antigenically distinct species of the immunomodulatory moiety lipoarabinomannan partiotioned in both aqueous and detergent phases. Human T cell proliferation assay showed a significantly greater potency of IMP over PMP. IMP subunits of <56kDa were resolved into 15 fractions using a continuous elution SDS-PAGE based protocol and all fractions, after SDS removal, were subjected to T cell proliferation assays for the identification of immunodominant subunits. Proteins falling within three low molecular weight zones (all <35kDa) did better than the rest, particularly the $\cong$ 22kDa protein which strongly stimulated T cells from all 5 donors. Partial overlap between IMP and secretory proteins, as noticed in this study, could proof clues to the immunodominance of the latter. The apparent uniquness of a high T cell activity potency make IMPs attractive candidates for designing future vaccines or immunotherapeutic agents.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 INTRODUCTION

1.1.1 Definition of problem

Mycobacterial diseases - tuberculosis and leprosy - have been causing enormous suffering to mankind for the past several millennia. Initially thought to be an evil of the tropical countries tuberculosis, now together with AIDS, has invaded the entire world. More disturbingly, the emergence of multidrug resistant strains of Mycobacterium tuberculosis is defying the achievements of past fourty years of effective chemotherapy. Prevention through effective vaccination is the ideal strategy for control and elimination of such diseases. However, till such an option is made available, early diagnosis and treatment are expected to restrict the disease prevalence.

BCG has extensively been used as a vaccine against tuberculosis and, more recently, against leprosy. The initial euphoria about its success has now vaned in the light of a variable efficacy seen in some recent major trials. The elucidation of mycobacterial constituents responsible for immuno supperssion, autoimmunity and inflammatory tissue damage has further weakened the propects for integral (whole cell based) vaccines and strengthened the case for rationally designed subunit vaccines.

Key to a successful subunit vaccine design lies in identification of immunodominant protein(s) capable of stimulating the appropriate subset(s) of T cells which are responsible for protection of the host against intracellular parasites. Knowledge about such proteins is also pertinent to the development of pathogen specific diagnostic tests. Despite the documentation of several such molecules, we are yet to identify a protein or subunit which could generate the T cell responses of desirable specificity and intensity in a cross-section of population.

An insight into the published work provides clues to the possible reasons for failures in picking the right molecule(s). First, despite the extensive use of molecular biology tools, only a handful of the antigenic constituents of mycobacteria have so far been described. The sequencing of M.tuberculosis and M.leprae genomes, which are nearing completion, highlight hundreds of open reading frames whose products

(proteins) are yet to be described biochemically as well as antigenically. A major limitation of the molecular cloning approach has been the availability of probes for selecting the gene products. The antigens have mostly been picked up with the help of monoclonal/ polyclonal antibodies which are available against only a limited number of specificities. The search for T cell inducing antigens is further constrained as all antibody reactive subunits need not be T cell reactive as well. The biochemical approach for identification and characterisation of mycobacterial antigens, as attempted by various laboratories, has also been restricted by nonavailability of appropriate tools which could ensure adequate resolution and recoveries of individual macromolecules from respective crude mixtures. Further, these attempts have been directed towards some but not all compartments of the microbe. Major efforts have gone into defining antigens of mycobacterial cytosol, cell wall and culture filtrates; but an antigenically important compartment- cell membrane - which is the focus of this study, has attracted least attention.

Considerable evidence exists that membrane proteins, especially the detergent soluble or 'integral' ones, of intracellular parasites are highly immunogenic. A recent report from our laboratory has shown, for the first time, that a collection of such potent human T cell stimulatory proteins does reside within the detergent soluble fraction of mycobacterial membranes (Mehrotra et al., 1995).

1.1.2 Objective

BCG, in the face of all controversies, still survives as the most widely used vaccine against tuberculosis and leprosy all over the world. None of the candidate vaccines put to human trials till date have been able to surpass it. Due to its safe usage and adjuvant properties, BCG is also being developed as a 'vehicle' for immunoprotective molecules of various pathogens. Its effectiveness against leprosy highlights the relevance of shared mycobacterial antigens in conferring protection. In this setting, an understanding of the immunodominant constituents of this vaccine is likely to provide clues to its effectiveness and a rationale for designing newer

immunoprophylactic or immunotherapeutic agents.

The present study aims at:

(a) antigenic definition of plasma membrane proteins, particularly the detergent soluble or 'integral' membrane proteins (IMPs), of BCG vaccine; and

(b) identification of the immunodominant T cell activating IMPs using a novel protocol for fractionation and screening of individual constituents.

Comparative evaluation of membrane proteins of BCG - a slow growing mycobacterium, with those of a 'fast grower species' (M. fortuitum) constituted a part of the strategy for achieving the above objectives.

The study is also likely to have implications for designing specific DNA probes based on partial sequences of the identified proteins. Such probes could serve as molecular biology tools for selecting proteins having direct relevance for T cells.

1.2 REVIEW OF LITERATURE

1.2.1 Mycobacterial diseases

Mycobacteria are intracellular parasites, having long been recognised as a scourge to the mankind. Existence of the two most prominent mycobacterial diseases, tuberculosis (referred to as 'white plague') and leprosy (as 'Hansens disease'), can be traced back to the prehistoric times. Even today, they figure amongst the most dreaded tropical diseases, particularly of the developing world.

Tuberculosis, caused by Mycobacterium tuberculosis (Koch, 1882), is the world's leading cause of mortality. One third of the world's population is considered infected, of which about 5% develop active disease. This represents 8 million new cases and 3 million deaths every year (Dolin et al., 1994).

Even the industrialized countries, after a century of progressive decline, are witnessing its resurgence in the wake of AIDS and emergence of multidrug resistant strains of M.tuberculosis. It has been declared 'global emergency' by world health

organisation. Statistics suggests that in the last decade of the 20th century an estimated 90 million cases of tuberculosis will occur resulting in over 30 million deaths - a toll greater than that of any other human pathogen (Bloom and Murray, 1992; Kochi et al., 1993; Dolin et al., 1994). Such statistics are clearly disappointing for a bacterial disease that until recently was considered one of the major triumphs of antibiotic revolution.

Leprosy is caused by Mycobacterium leprae (Hansen, 1955) which is closely related to M. tuberculosis. The parasite has affinity particularly for skin and nerves. In contrast to tuberculosis, leprosy does not have high mortality but is associated with horror and fear due to the deformities and crippling caused by it in approximately 30% of the population. Implementation of multidrug therapy program by WHO (WHO, 1982) has reduced world wide prevalence of leprosy by more than 80% since 1985, from 10-12 million to 1.8 million patients in 1995 (Noordeen, 1995). Yet new case detection remains at a high level; 414,894, new patients were detected in India alone during 1994 (Noordeen, 1995; WHO, 1995). Therefore, the current success in leprosy control must not lead to the type of complacency in research that was witnessed in the case of tuberculosis by an early success of chemotherapy.

1.2.2 Treatment of mycobacterial diseases

Monotherapy based on a simple sulphone, dapsone was successful for treatment of leprosy for almost two decades (Waters, 1983). It was only in 1977 that multidrug therapy with bactericidal drugs (dapsone, rifampicin, clofazimine and ethionamide) comparable to the treatment of tuberculosis was recommended for treating all cases of lepromatous leprosy and subsequently for the paucibacillary cases too (WHO, 1982).

A triple - drug regimen was recommended for treatment of multibacillary leprosy (LL, BL and BB) patients with a minimum duration of 2 years and which should routinely be given until the patients become smear negative. Short course chemotherapy with monthly rifampicin and daily dapsone in paucibacillary leprosy (TT and BT) for a total of 6 months appears very effective although long - term follow

up is essential.

Since the first official use of streptomycin in 1947, five drugs have emerged as first line agents for the treatment of tuberculosis: isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin. Tuberculosis drug regimens are divided into two phases: the intensive phase of treatment during which the aim is to kill the population of bacilli that are dividing, and the continuation phase during which the aim is to kill bacilli that are more dormant. Current strategies center on treatment with multidrug regimens based on the very effective combination of rifampicin and isoniazid. Major obstacles to the success of chemotherapy are difficulty in early diagnosis and low patient compliance. The latter is a result of long treatment regimens and social concerns. The problem is further compounded with the occurrence of HIV and emergence of multidrug resistant strains of M. tuberculosis (Kochi et al., 1993).

The present concern is to explore strategies for new drug development by identifying new targets within the pathogen (Young and Duncan, 1995). Most of the available antimycobacterial drugs act by inhibiting biosynthetic pathways involved in production of macromolecules. For example streptomycin interferes in protein synthesis; dapsone in synthesis of nucleic acids and cycloserine; isoniazid, ethambutol and ethionamide in cell wall biosynthesis; the fluoroquinolones are the broad spectrum antibacterial agents which disrupt bacterial chromosome by inhibiting supercoiling activity of DNA gyrase. Rifampicin is the key drug having broad antibacterial spectrum by inhibiting transcription. The complex cell wall structure of mycobacteria acting as a permeability barrier for many existing drugs also provides a unique structure containing several potential drug targets viz., the arabinogalactan and the unique mycobacterial mycolic acids. Most of the enzymes responsible for synthesis of cell wall components would probably reside in the mycobacterial cell envelope thereby, rendering important, the analysis of cell membrane proteins.

1.2.3 Mechanism of protective immunity against mycobacteria

1.2.3.1 An overview of immune response

Successful immune response to an infectious agent depends on the activation of an appropriate set of immune effector functions, which can broadly be divided into two types: cell mediated and humoral. Cell mediated immunity (CMI) involves induction of T cells and activation of macrophages, whereas, humoral immunity (HI) is characterised by activation of B cells and antibody production.

Factors involved in determining the type of immune response are under intensive investigation (Fig.1.1). $CD4^+$ T cells recognise antigens presented by class II MHC (major histocompatibility complex) molecules which are preferentially derived from phagocytic vesicles of the antigen presenting cell (APC, eg., macrophages). Activation of $CD8^+$ T cell requires presentation of antigens by class I MHC molecules derived from the cytoplasm of APC (Germain and Margulies, 1993). Extensive investigation on $CD4^+$ as well as $CD8^+$ cells show that during activation they become committed to expression of one of the two distinct sets of cytokines (Salgame et al., 1991). Cytokines that promote macrophage activation (eg., IFN-γ) characterize the Th1 response, whereas Th2 cytokines (eg., interleukin-4) promote antibody formation at the expense of macrophage activation. The balance between Th1 and Th2 responses is influenced by the local cytokine environment at the early stage of T cell activation (Hsich et al., 1993). The key cytokines include IL-12, which promote Th1 responses and IL-10, which favours Th2. Both cytokines are secreted by macrophages (as well as other cell types) in response to bacterial constituents.

The mononuclear phagocyte (macrophage) is a potent antimicrobial component of CMI. Some of the best charactrerized antimicrobial effector functions of macrophage are phagosome lysosome fusion and generation of reactive oxygen/nitrogen intermediates (Kaufmann, 1993). Apart from being principal effectors, macrophages also provide a preferred biotype for mycobacteria thereby promoting survival of the pathogen. Similarly T cell (eg., Th2 subset) also contribute

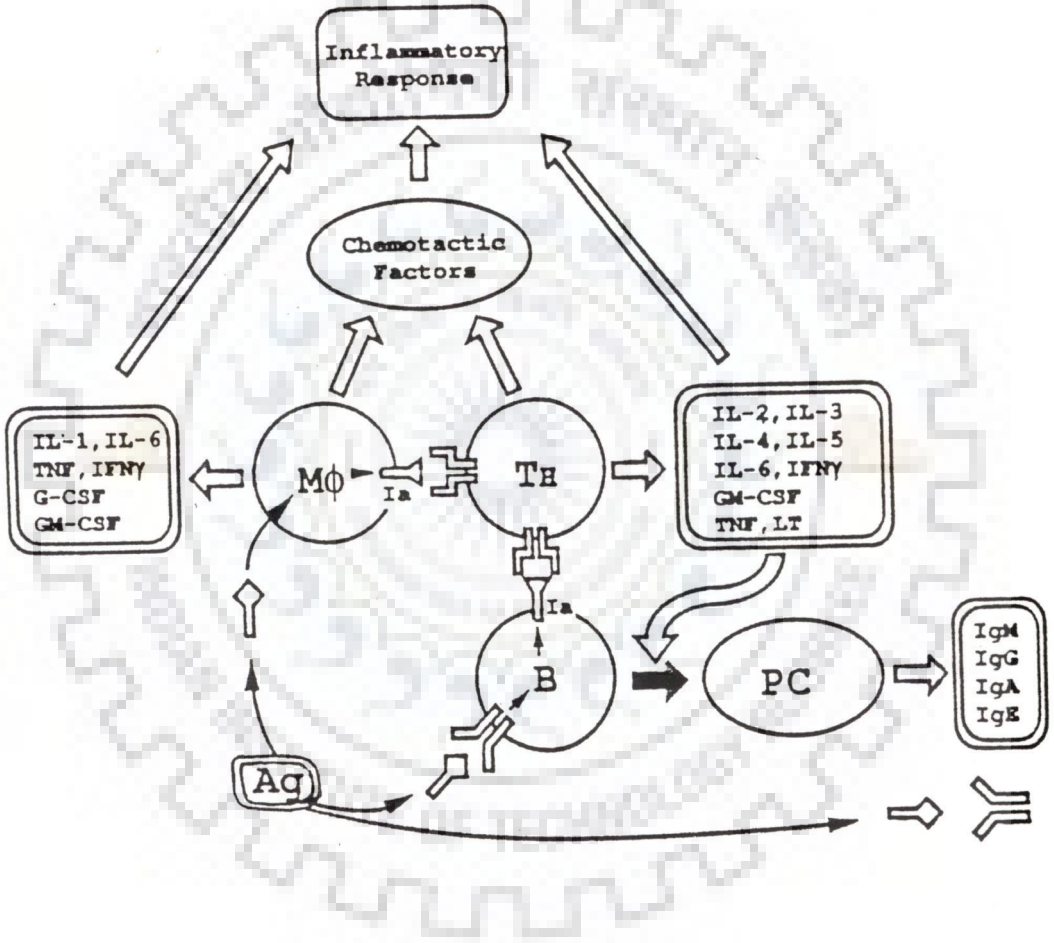

Fig. 1.1 Role of T cell and macrophage-derived cytokines in immune and inflammatory responses.

to pathogenesis. A coordinated cross-talk between the two is essential for protection.

1.2.3.2 Immunity against mycobacteria

Mycobacteria and their constituents elicit both humoral and cell mediated immune responses. Antimycobacterial antibodies are known not to confer passive protection, although their contribution to pathogenesis has not been ruled out. They are found in abundance even in patients having a generalised infection (Ivanyi et al., 1988).

Acquired resistance against mycobacteria depends on CMI with major effectors being mononuclear phagocytes (macrophages) and T lymphocytes. An effective T cell response is mandatory for protection against mycobacterial diseases. Two functions are currently envisaged for T cells involved in protective immune responses (Kaufmann, 1993). The cytotoxic (Tc) function is required to release intracellular mycobacteria which establish themselves within macrophages (or other cell types in the case of M.leprae, whereas the helper (Th) function is required to recruit and activate new monocytes or macrophages to the site of lesion. The Th1 cells bearing $CD4^+$ phenotype probably play the central role in macrophage activation mediating their effect via interferon-γ(IFN-γ), a key cytokine secreted by them (Flynn et al.,1993). $CD8^+$ T cells express specific cytolytic activities which have been shown to be critical in protection using gene knock out mice (Flynn et al., 1992). However, recent evidences have indicated that the 'cytolytic' activity could also be an attribute of $CD4^+$ and 'double negative' ($CD4^-$, $CD8^-$) T cells (Stenger et al., 1997; Mutis et al., 1993). Moreover, the $CD8^+$ T cells are now believed to contribute mainly by way of IFN-γ production rather than cytolysis (Stenger et al., 1997; Condos et al., 1997). Another subset of lymphocytes, characterised by an antigenic receptor composed of γ and δ chains also secrete IFN-γ and may be important triggers at the initial stage of immune response (Janis et al., 1989).

1.2.4 Immunopathology of Leprosy and Tuberculosis

Leprosy is characterised by a spectrum of symptoms, largely a result of the

immunological responses of the host to the antigens of M.leprae.

A major breakthrough was achieved by Ridley and Jopling (1966) in the classification of leprosy according to clinico-pathological observations. At one pole (lepromatous leprosy, LL) there is an enormous growth of the bacilli in the tissue resulting from a specific and controlled lack of cell mediated immunity (CMI) of the host to M.leprae. At the opposite pole (tuberculoid type, TT) patients are responsive to M.leprae antigens, and there are few bacilli present in the lesions. Intermediate forms comprise "borderline lepromatous" (BL) "midborderline" (BB), and "borderline tuberculoid" (BT) leprosy.

Many clinical and histological observations have suggested that a spectrum of immune response, like leprosy, occurs in tuberculosis also. An analysis of the immunological spectrum in tuber culosis was undertaken by Lenzini, Rottoli and Rottoli (1977). According to them, tuberculosis patients can be classified into four groups: reactive (RR), reactive intermediate (RI), unreactive intermediate (UI) and unreactive (UU). The patients were assessed by means of clinical and radiological data, response to chemotherapy, skin delayed hypersensitivity testing, histology of dissected lesions, leucocyte migration test and the presence of antimycobacterial antibodies. The unreactive (UU) form is some- what analogous to lepromatous leprosy but the organism M.tuberculosis is far too toxic to permit a chronic lepromatous form of TB to exist.

1.2.5 Structure of mycobacterium

Mycobacteria are gram positive, non-motile, non-sporeforming, pleomorphic rod shaped microbes. They range from nonfilamentous to filamentous types. Electron microscopy shows that mycobacterial cell envelope consists of two distinct parts: plasma membrane and, around it, a thick cell wall. Both the parts are separated by a narrow electron transluscent zone, termed periplasmic space.

Cell wall itself is a complex structure composed of four layers (Fig. 1.2). The

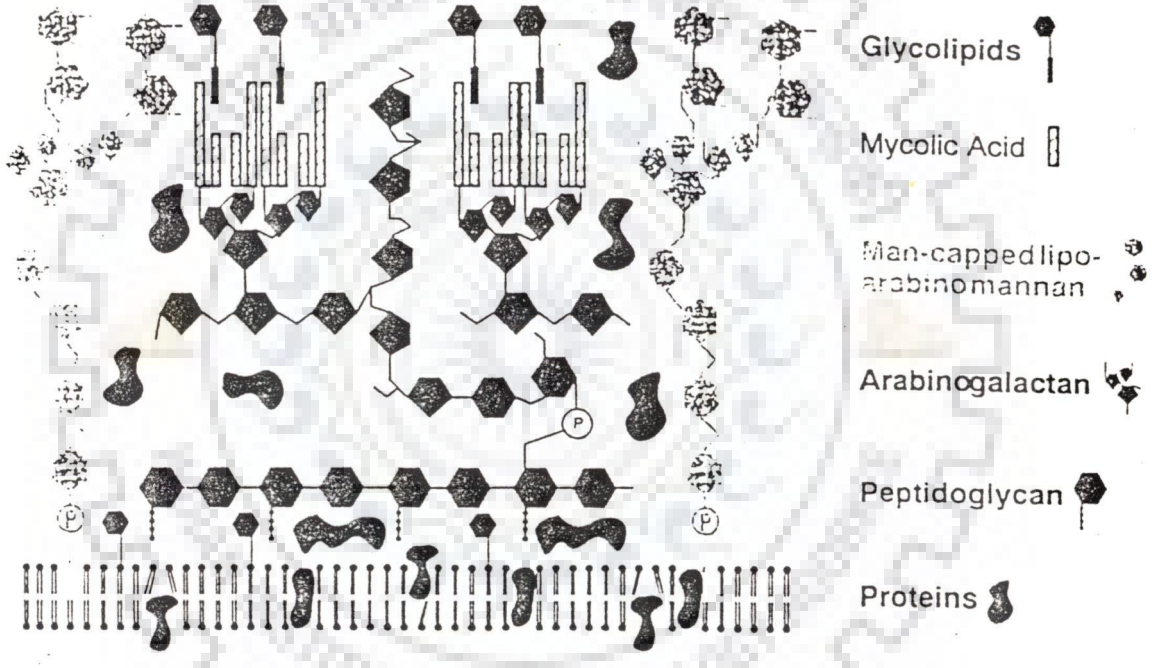

Fig. 1.2 Schematic representation of a mycobacterial cell envelope showing : (a) the lipid bilayer of plasma membrane with integral (embeded) and peripheral (loosely attached) proteins and (b) cell wall constituents.

innermost layer consists of murein or peptidoglycan whose chemical structure closely resembles that of other bacteria. Superficial to the murein layer are three fairly distinct layers composed of rope like complexes of peptides, polysaccharides and lipids set in a more homogeneous matrix. The structures in the three layers differ chemically. Those in the outer layer are principally peptidoglycolipids termed mycosides (Barksdale and Kim, 1977). The murein layer consists essentially of molecules of N-glycolyl muramic acid and N-acetylglycosamine linked alternatingly in a linear fashion. Crosslinking is provided by short aminoacid chains, containing alanine or glycine. This net like structure is cleaved by the enzyme lysozyme. One of the products of this enzyme cleavage is muramyl dipeptide (MDP, N-acetyl muramyl-L-alanyl-D-isoglutamine), a powerful immunological adjuvant (Nagaoet al., 1981).

Membranes of mycobacteria appear in ultrathin sections as classic bilayers, with the two electron dense layers separated by a transparent layer (Silva and Macedo, 1983). This appearence, coupled with their chemical composition (Kumar et al., 1979) indicates that these are "normal" biological membranes.

The cytoplasm of mycobacteria does not differ essentially in the fine structure from that of other bacteria. DNA is observable as a rather ill-defined nuclear body which, in common with all other bacteria, is not bound by a membrane. Other particles seen in cytoplasm include ribosomes, lipid bodies and granules composed of polyphosphates which probably serve as energy stores.

1.2.6 Immuno/pathologically relevant constituents of mycobacteria

Based on their preferred locations, mycobacterial antigens can be categorised under 4 heads (i) extracellular/secretory antigens; (ii) cell wall antigens; (iii) membrane antigens and (iv) cytosolic antigens (Thole et al., 1995; Young et al., 1992).

1.2.6.1 Extracellular/ secretory proteins

These are proteins released into the culture medium by viable mycobacteria early during growth and may be amongst the first antigens encountered by the immune

system. However, they are considered important for eliciting a protective immune response.

The 30/31 kDa antigen 85 complex is a major component of the culture filtrates of M.tuberculosis, M.leprae and BCG (Wiker and Harboe, 1992; Launois et al., 1995; Fifis et al., 1991). Three members of this family which have approximately 80% amino acid sequence identity amongst each other are designated as 85A, 85B and 85C. Recently a fourth distantly related member of 27 kDa has also been identified which displays 40% homology with the antigen 85 complex (Rinke de Wit et al., 1993). These proteins bind to fibronectin coated surfaces, mediating uptake of mycobacteria by host (Thole et al., 1992). But recently, this biological role of antigen 85-complex in M.leprae has been questioned (Pessolani and Brennan, 1992). It has been characterised as mycolyl transferase assisting the biosynthesis of mycolic acids of mycobacterial cell wall (Besra et al., 1994). The protein has been assessed for its diagnostic potential by several workers. It generates high antibody titres in tuberculosis patients (Barnes et al., 1989). Three epitopes of M.leprae antigen 85B molecule have been identified which are recognised exclusively by leprosy patients (Filley et al., 1994). Antigen 85 has also been shown to induce a Th1 type of response in tuberculosis patients (Launois et al., 1995).

A 24 kDa protein of M. tuberculosis is another major component of secreted products. Initially isolated from M.bovis BCG culture filtrate and designated as MPB64 (Harboe et al., 1986), the protein elicited strong DTH reaction in guinea pigs immunised with mycobacteria belonging to M. tuberculosis complex only. This specificity is surprising because the protein is detected in M.kansasii also (Andersen et al., 1991). Different substrains of BCG have shown different levels of expression of this protein.

A novel low mol wt 6 kDa protein designated as ESAT-6 (early secretory antigenic target) has been purified from M.tuberculosis (Sorensen et al., 1995). The protein has been demonstrated to be one of the major targets of memory effector cells in mouse

model. It is currently being investigated as a potential vaccine.

A 33/36 kDa'proline rich' antigen described in M.leprae has shown impressive serodiagnostic potential with high specificity and sensitivity for leprosy (Thole et al., 1990). A protein homologous to this antigen has been isolated from BCG culture filtrate (Romain et al., 1993).

The 19 and 38 kDa proteins detected in the culture filtrates of M.tuberculosis have been characterised as lipoproteins (Daniel and Andersen, 1978; Young and Garbe, 1991) and are therefore, also found associated with the cell envelope. The 19 kDa protein has also been detected in the culture filtrate of BCG (Fifis et al., 1991). Both the proteins are glycosylated and induce a Th2 type of immune response (Surcel et al., 1994). The 38 kDa antigen has been found useful for sensitive and specific serodiagnosis of tuberculosis (Harboe and Wiker,1992). Despite unimpressive human T cell activation by both these antigens (Surcel et al., 1994), the 38 kDa antigen(also known as antigen 5) was evaluated as a diagnostic skin test reagent in large scale clinical trials without desired success (Daniel et al., 1982). Based on its sequence homology with E.coli PstS (phosphte transport protein), the 38 kDa antigen is suggested to have a role in phosphate transport (Andersen et al., 1990).

1.2.6.2 Cell wall antigens

Carbohydrates and lipids

The carbohydrates and lipids of the mycobacterial cell wall can be described under following entities: peptidoglycan, arabinogalactan and glycolipids (Fig. 1.2).

Peptidoglycan: The peptidoglycan polymer which confers rigidity to bacterial cell walls, is built of glycan chains with alternating N-acetylglucosamine and N-acetyl muramic acid residues which are linked through peptide side chains (Fig. 1.2). Variations occur in the composition of aminoacids as well as in the degree and nature of cross-linking. Adjuvanticity, reflecting the potency of substances to enhance the antibody response to antigens, is a well known property of mycobacteria. Degradation

and synthetic studies have delineated that this adjuvant property is associated with 'muramyl dipeptide' or MDP (N-acylmuramyl-L-alanyl-D-isoglutamine). MDP exerts multiple immunopharmacological activites, e.g., macrophage stimulation with or without participation of T cells (Nagao et al., 1981).

Arabinogalactan : It is a major polysaccharide constituent representing overt antigens of the cell wall built up of galactofuranose and arabinofuranose residues. It is linked to the peptidoglycan polymer via a unique diglycosyl-phosphoryl bridge containing L-rhamnose and N-acetyl-glucosamine. The arabinogalactan of M.leprae and M.tuberculosis contains exclusively arabinofuranosyl and arabinogalactosyl residues (Brennan and Nikaido, 1995). Towards the outer surface these arabinogalactans are bound to mycolic acid esters which are long chain branched fatty acids containing 60-90 carbon atoms making up the largest single component of the mycobacterial cell wall. They probably represent the structure responsible for granuloma/tubercle formation, a characteristic pathology of mycobacterial lesions (Emori et al., 1985). Such lesions are much less apparent in other bacterial infections where presence of MDP is nearly ubiquitous.

Glycolipids : Phenolic glycolipid (PGL-1) is the abundant surface glycolipid unique to M.leprae. It is detectable in tissues and serum of lepromatous leprosy patients. The terminal trisaccharide is the target of a species specific immunoglobulin - M (IgM) antibody response which is maximal at the lepromatous pole of the disease. It has been used in the serodiagnosis of leprosy (Cho *et* al., 1983). It is known to modulate the monocyte/ macrophage function by acting as a scavenger of OH·, thereby protecting the organisms from the toxic oxidative burst (Neill and Klebanoff, 1988).

Lipoarabinomannan (LAM) is highly antigenic mycobacterial equivalent of lipopolysaccharide (Brennan and Nikaido, 1995) consisting of repeating units of arabinose and mannose anchored via a phosphatidylinositol moiety to bacterial membrane (Fig. 1.2). LAM stimulates a cross-reactive antibody response in leprosy and tuberculosis patients and has implications for serodiagnosis (Miller et al., 1983). LAM

from both M.leprae and M.tuberculosis is associated with broad immunosuppressive effects which contribute to pathogenesis of the disease. It is known to suppress T cell proliferation (Moreno et al.,1988), to inhibit γ-interferon mediated activation of macrophages (Sibley et al.,1990) and to enhance production of tumor necrosis factor (TNF) by mononuclear cells (Chatterjee et al.,1992). Structural heterogenicity in LAM from different mycobacterial species or even from the same species has been documented (Leopold and Fischer,1993). It is not clear how these structural variations determine the immunomodulatory properties. Recently LAM of M.tuberculosis has also been shown to possess chemotactic activity (Berman et al.,1996).

Proteins

Important protein-based immunoreactvities (especially cell mediated ones) have long been associated with the cell walls of mycobacteria (Kaplan et al.,1988). Various cell wall fractions have been shown to confer protection in mouse against challenge with M.leprae (Gelber et al.,1990). Proteins associated with the peptidoglycan of M.leprae have been shown to stimulate T cells from patients with tuberculoid leprosy and also DTH responses in guinea pigs (Kaplan et al.,1988; Hunter et al.,1989). The soluble proteins that integrate into the cell wall skeleton have been characterised using specific T-cell clones from leprosy patients. Greatest T cell reactivites have been observed with 7 kDa, 16 kDa and 28 kDa antigens (Mehra et al.,1989).

The task of dissociating proteins from the insoluble peptidoglycan complex was formidable. However, a 23 kDa protein has been characterised as a major constituent of the cell wall protein-peptidoglycan complex in M.tuberculosis. The protein shows homology with the outer membrane protein (Omp F) of E.coli. In view of the apparent hydrophobicity of the protein and its absence from culture filtrate and cytosolic or membrane fractions, it is suggested to have a role in solute transport through the lipid barrier of mycobacteria (Hirschfield et al., 1990).

1.2.6.3 Membrane proteins

Two major membrane proteins described from M.leprae are 18 kDa and 35 kDa (Hunter et al.,1990).These proteins were isolated from the aqueous phase after extracting the membrane with Triton X-114 detergent. The 35 kDa protein contains M.leprae specific antibody epitopes (Ivanyi et al., 1983) and T cell epitopes (Mohagheghpour et al., 1990). It has proved a promising candidate for diagnosis of leprosy (Sinha et al., 1983; Triccas et al., 1996). The 18 kDa protein is a bacterioferritin involved in iron metabolism possibly playing the role of a virulence factor (Pessolani et al.,1994).

A 19 kDa water soluble membrane protein of M.tuberculosis is a novel antigen capable of eliciting anitibody responses (Lee et al., 1992).

A 46 kDa membrane protein of M.leprae designated as 38L is responsible for the transport of small molecules such as tyrosine across the membrane (Oskam et al.,1995). It shows remarkable homology with the pink-eyed dilution protein (P protein) of human and murine origin responsible for hypopigmentation observed in leprosy patients.

A 45 kDa membrane protein is recognised specifically by leprosy patient sera (Vega-Lopez et al., 1993).

1.2.6.4 Cytosolic proteins

Stress proteins (Heat Shock Proteins)

These proteins belong to the highly conserved family of heat shock proteins (hsp) in prokaryotes and eukaryotes which serve multiple functions. An important one is to assist the microorganism in adaptation to environmental changes by acting as "molecular chaperons" mediating folding, assembly and translocation of proteins. Though they are also present in unstressed cells, their levels increase markedly under environmental or host-induced changes viz., elevated temperatures, oxygen radicals, metal ions etc.

The extensively studied mycobacterial 70 kDa, 65 kDa and 10 kDa hsps are homologous to the evolutionarily highly conserved E.coli Dnak, GroEL and GroES, respectively. Despite extensive sequence identity with mammalian homologues, they stimulate strong cellular immune responses in patients or their contacts (Mehra et al.,1996). However, their association with autoimmune disorders has reduced interest in these antigens from vaccine point of view (Young,1990).

The 18 kDa protein of M.leprae which shares partial identity with low molecular weight hsps, has been shown to stimulate peripheral blood T cell and antibody responses in leprosy patients (Dockrell et al., 1989; Roche et al.,1992).

A 14/16 kDa protein of M.tuberculosis is homologous to the α-crystallin family of low mol wt hsps. The protein has shown serological as well as T cell stimulatory activity (Vordermeier et al., 1993).

Enzymes

Mycobacterial superoxide dismutase (SOD), seems to have a distinct function of paralyzing the host defence mechanisms by inactivating the toxic superoxide radicals generated by activated macrophages (Fridovich, 1972). The protein is exported to the exterior (therefore, also characterised as secretory protein) but does not contain a consensus signal sequence. Its subunit size in M. leprae and M.tuberculosis is 28 kDa and 23 kDa respectively, (Young et al., 1985 ; Kusunose et al., 1976). Mycobacterial SODs bear considerable structural homology to the human mitochondrial SOD. Its deposition in skin lesions of about 55% leprosy patients suggests its immunopathalogical relevance (Khanolkar et al., 1989). The native mycobacterial SOD, purified from the vaccine candidate M. habana, has been shown to be a potent inducer of T cells from experimental animals as well as humans (Bisht et al., 1996).

A 49 kDa protein from M.leprae is a hybrid protein comprising both thioredoxin-reductase as well as its substrate, thioredoxin linked together via a spacer (Wieles et al., 1995). In all other prokaryotic and eukaryotic organisms studied till date these

enzymes are encoded by separate genes. Together with NADPH they form an integrated system providing electrons to a variety of metabolic processes. Thioredoxin may also have a role in reduction of reactive oxygen species (Mitsui et al., 1992).

A 40 kDa protein of M.tuberculosis was identified by sequence homology and functional analyses to be an L- alanine dehyderogenase (Andersen et al., 1992).

1.2.7 Role of mycobacterial antigens in designing control strategies

Effective vaccination is the ideal strategy for primary prevention and eradication of tuberculosis and leprosy. In its absence, early diagnosis and treatment are expected to control the progression and transimission of the infection.

1.2.7.1 Diagnosis

Early diagnosis of mycobacterial infection mainly relies on identification of the organism in patients along with clinical, pathological or radiological parameters. Conventional microbiological identification of mycobacteria involves acid fast staining of patients sputum (in case of tuberculosis) or skin (in case of leprosy)smears. The assay lacks sensitivity and specificity and is mostly negative in the paucibacillary cases of leprosy and tuberculosis. Development of effective diagnostic assays has been of utmost concern to the researchers. A number of such assays have been developed till date which are based mainly on the humoral (antibody) or T cell mediated (delayed type hypersensi-tivity, DTH) responses.

Serodiagnosis

The diagnostic potential of a serological test based on detection of antigen or antibody in the patient is determined by its specificity (lack of 'false positives') and sensitivity (lack of 'false negatives'). Antigen detection assays have shown lesser practical utility than the antibody detection assays, since the former require high bacillary load for which large amounts of samples (tissues or body fluids) need to be collected. On the other hand, widespread use of BCG vaccine has undermined the utility of antibody based assays particularly in tuberculosis due to its antigenic closeness

to the pathogen.

For a single mycobacterial antigen to be useful as a diagnostic reagent, it should be specific for the pathogen. The search for such antigens has received a boost, albeit limited, with the success of affinity purification and recombinant techniques.

Detection of specific antigen/ antibody in leprosy and tuberculosis

(a) Detection based on polyclonal antibodies

Several sensitive ELISA based assays involving specific antigens and antibodies of M.leprae have been developed (Anonymous, 1986). The most popular and widely accepted of these is based on the cell wall associated specific carbohydrate phenolic glycolipid-I (PGL-I) (Cho et al., 1983). The anti-PGL-I antibody assay is more sensitive than the corresponding antigen assay. It puts to evidence 95% of multibacillary (MB) cases but lowers to 30% in detection of paucibacillary (PB) cases (Cho et al., 1984). Besides diagnosis, the assay has found utility in monitoring chemotherapy (Chanteau et al., 1989).

Unlike leprosy where success in serodiagnosis was achieved on the basis of a whole molecule (PGL-I) which is found exclusively in M.leprae, trials with various well characterised antigenic proteins did not lead to success in serodiagnosis of tuberculosis. ELISA with antigen 5, the well characterised 38 kDa protein did not show desirable specificity (Daniel and Debanne, 1987). Similarly, the use of purified antigen 85 (_30/ 31 kDa) was disappointing on both counts - sensitivity and specificity (Wiker and Harboe, 1992). The previously thought serologically promising 14/16 kDa antigen was later found to be present in the M.tuberculosis complex (M.tuberculosis, M.africanum, M.bovis and M.microti) (Verbon et al., 1993).

Thus, there is absolute necessity to identify antigens which could discriminate between active tuberculosis and BCG vaccination. Crossed immunoelectrophoretic studies (Closs et al., 1980) have shown that most antigens found in M.bovis (BCG) are also present in M.tuberculosis, but no information is available to suggest that the

converse is also true. Higher expression of epitopes of 38 kDa protein in M.tuberculosis as compared to M.bovis has been reported (Young et al., 1986). In a recent study, immunoblots of one and two dimensional SDS-PAGE of antigenic extracts of M.tuberculosis and M.bovis BCG with tuberculosis and BCG-vaccinated control human sera could be distinguished by analysing the reactivity of the two to a combination of antigens in different molecular mass ranges (Bassey et al., 1996).

(b) Detection based on monoclonal antibodies (moAbs)

The realisation that species-specificity of mycobacterial antigens almost certainly rests not with the whole molecules but their epitopes (Daniel et al., 1986) led to the production of moAbs against various mycobacterial antigens and identification of cross-reactive and specific epitopes therein for subsequent use of the latter in serological assays based on ELISA.

Solid-phase antibody competition test (SACT) was developed (Hewitt et al., 1982) which is based on inhibition of binding of labelled moAb to antigen by antibodies present in the test sera. The test is carried out using microtitre plates coated with a crude soluble extract of M.tuberculosis (MTSE) or M.leprae (MLSE). The test has provided well defined discrimination between healthy and infected individuals.

The moAb based assays have had particular relevance to the detection of leprosy due to the availability of a panel of moAbs unique to M.leprae (Engers et al., 1985). SACT based on species specific MLO4 epitope of 35 kDa protein has been used for detecting leprosy (Sinha et al., 1983). It is more sensitive than PGL-I based ELISA. Various other anti-M.leprae moAbs have also found utility in these tests (Klaster et al., 1985).

Less success of moAb based assays has been achieved in diagnosis of tuberculosis because majority of them fail to distinguish between active infection and BCG vaccination (Engers et al., 1986). The 38 kDa and 14 kDa antigens of M.tuberculosis were reported to carry epitopes restricted to M.tuberculosis complex. Binding assay

with 38 kDa protein showed a positive correlation with antibody titres measured by competition with moAb TB72, which binds to the immunodominant epitope, as well as the absence of antibody to this antigen in control sera (Young et al., 1986). Although highly specific, the test has low sensitivity (Jackett et al., 1988).

DTH based tests

DTH synonymous to Koch's phenomenon is a class of T cell mediated immune response leading to macrophage activation and is exemplified by a characteristic skin-test. An intradermal injection of mycobacterial antigens attracts the previously sensitized T cells ($CD4^+$) to the skin test site which proliferate producing cytokines, thereby activating the macrophages leading to an induration or erythema at the site of injection which is manifested after 24 to 48 hrs.

Tuberculin test

DTH response to M.tuberculosis is evaluated by tuberculin skin test (Mantoux) involving intradermal injection of PPD (purified protein derivative) prepared by ammonium sulfate precipitation of M.tuberculosis culture filtrate (Seibert and Glen, 1941). It is popularised under the name RT 23 by WHO as the international standard for tuberculin. It is estimated that some 2 billion doses of RT 23 have been administered world wide to humans, and is still being widely used. But the test lacks specificity because PPD contains many antigens that are shared with non-tuberculous environmental mycobacteria, as well as BCG. Sensitization with these organisms yields a positive test in the absence of tuberculous infection. However, the size of induration can offer some discrimination between active infection and sensitization.

Lepromin test

The classical way to skin test leprosy patients is lepromin, which is a heat-killed suspension of M.leprae (40 million bacillary suspension/ml in normal saline). The test is read at 48-72 h (Fernandez reaction) and again at 21-28 days (Mitsuda reaction). The former probably represents a phenomenon analogous to a Mantoux test, caused

by soluble components of lepromin (though it is never necrotic). The late 'Mitsuda reaction' is considered more specific for leprosy. The latter reaction becomes apparent after about seven days, and may develop into a nodule 1 cm in diameter within three to four weeks. Intact bacilli are important to the Mitsuda reaction. If these organisms are disrupted, the resulting soluble antigen (leprosin) evokes only a 24-48 hours skin reaction (Rook, 1983).

Although a lepromin test has no diagnostic potential, it has considerable prognostic value and provides confirmatory evidence for classification of the disease. So lepromin has been used to determine the CMI status of patients classified by Ridley-Jopling scale. In most of the TT/BT cases the test is strongly positive, while in BL/LL cases it is negative. However, the negative skin-reaction of the lepromatous cases is remarkably specific for M.leprae, because the response to other mycobacterial antigens including Mantoux test is normal.

Other antigens evaluated in skin test

The realisation that T cell based diagnosis could be more sensitive than serodiagnosis has generated a quest for identification of antigens or subunits which would induce pathogen specific skin DTH responses.

Antigen 5 (38 kDa protein) was shown to be limited in distribution to M.tuberculosis and BCG and to be a satisfactory tuberculin actigen in experimental animals (Daniel et al., 1979). However, in large scale clinical trials with native 38 kDa protein, the initially generated high hopes remained unsubstantiated. It was found to offer no advantage over PPD (Daniel et al., 1982).

The 24 kDa (MPT-64)and 32 kDa (MPT-59) secretory proteins of M.tuberculosis which also showed initial promise in animal studies (Haslov et al.,1995), were not found superior to PPD in clinical trials (Wilcke et al., 1996).

Fractionation and identification of M.leprae antigens which could provide a more

sensitive and specific skin DTH compared to lepromin are underway. MLSA-LAM (M.leprae soluble antigens without LAM) and MLCWA (M.leprae cell wall antigens) are slated for clinical trials (Brennan et al., 1996).

The 35 kDa M.leprae specific antigen is a promising candidate for developing a diagnostic skin test for leprosy. It elicits only a weak response in BCG or M.tuberculosis sensitized animals (Triccas et al., 1996).

DNA based diagnosis

Advances in molecular biology and biotechnology have created exciting possibilities for DNA - based diagnosis (Kohne. 1989). Molecular hybridisation of a radio-labelled or chemiluminiscent DNA probe (a short oligonucleotide sequence based on a specific sequence in the pathogen DNA) with the target DNA is used . The dot blot DNA hybridisation technique is simple and can be used for large scale screening. Chemiluminiscent DNA probes have been used in the detection of tuberculosis (Evans et al., 1992). A further advancement in these tests has involved amplification of target DNA by polymerase chain reaction (PCR) thus enabling detection of few bacilli in just a few hours. They have been used in the detection and identification of M.tuberculosis and M.leprae (Brisson-Noel et al., 1989; Eisenach et al., 1990; Hance et al., 1989; Plikaytis et al., 1990). Most published PCR - based tests have been shown to work well in the laboratory. Three recent tests could be transferred to clinical laboratory. The specificity of these assays was 100% though their sensitivity was less as compared to that in the research laboratory. It is still to be seen whether these assays could have field utility in a cost effective manner.

1.2.7.2 Vaccination

Vaccination is generally used as a form of immunoprophylaxis for the primary prevention of a disease. Administration of a vaccine mimics natural infection, thereby generating a life long memory of the infectious agent in the host without exposing the

latter to the risk of developing a disease.

Integral Vaccines

BCG

The observation that exposure to tubercle bacilli develops disease only in a few individuals while rendering others resistant to fresh infection, initated attempts to attenuate the organism. In the 1920s, Calmette and Guerin, succeeded in attenuating the bovine tubercle bacillus (Mycobacterium bovis) by sequential culture on potato bile broth, a process repeated 230 times over 13 years which resulted in the vaccine, BCG (Bacillus Calmette Guerin).

BCG was first used to protect against tuberculosis in 1921. Since then it continued to be propagated by subcultures for 40 years in various laboratories under a variety of culture conditions. Over the past 60 years some 3 billion doses of BCG have been administered world over. However, some major recent trials have put its use to question due to the wide variability in protective efficacies seen in different parts of the world (Ponnighaus et al., 1992). A recent trial in south India calculated its efficacy close to zero (editorial, 1980). A general consensus is that BCG vaccination consistently protects against primary, disseminated form of tuberculosis (Rodrigues et al., 1993),but protection against pulmonary disease varies widely in different trials (Fine et al., 1990; Bloom and Fine, 1994). Results pooled from a range of trials indicate an overall reduction of 50% in the risk of tuberculosis following BCG vaccination (Colditz et al., 1994). These data are consistent with the idea that BCG vaccination effectively boosts the immune response during primary infection but has limited effect on the subsequent course of dormancy and reactivation. Due to declining protective efficacy with time, after BCG vaccination, little or no protection is seen after 10-15 years (Rodrigues et al., 1993) suggesting that child hood vaccination may not prevent adult reinfection (Styblo and Meijer, 1976).

Ironically, the current perception is that BCG offers greater protection against

leprosy than tuberculosis (Malawi, 1996). The effectiveness of BCG against leprosy has generally been modest (Erwin et al., 1985) with the exception of a trial in Uganda (Stanley et al., 1983).

Apart of its vaccine value against mycobacterial diseases, childhood immunisation with BCG has recently been found to protect against atopic disorders (Shirakawa et al., 1997).

Reasons for variable performance of BCG in different trials have been attributed to certain factors related to the vaccine as well as the host (Table 1.1).

Other integral vaccines

Use of an M.leprae based vaccine was impeded by the inability to cultivate the bacilli in-vitro. Such a vaccine became feasible when it could be grown in armadillos (Kirchheimer and Storrs, 1971) and mouse foot pad (Shepard, 1960). A killed M.leprae based vaccine was put to trial in Norway in 1983 (Gill et al.,1986). A large scale trial to compare the protective effect of killed M.leprae+ BCG and BCG alone began in Venezula in 1984 and Malawi in 1985. The results of these trials give no evidence of better protection using M.leprae+ BCG than BCG alone (Fine and Rodrigues, 1990; Convit et al.,1992; Malawi Karonga, 1996).

A combination of killed M.vaccae (a rapidly growing non-pathogenic mycobacterium) with BCG has been found to show better efficacy than BCG alone (Stanford, 1990).

Therapeutic efficacy of another candidate vaccine Mycobacterium w as adjunct to multidrug therapy has been demonstrated in a number of studies (Talwar et al., 1990). In view of the encouraging results, Phase III immunoprophylactic trial of this vaccine has been initiated in India (Kanpur Dehat, March 1990).

ICRC vaccine is being used in patients of leprosy since 1979 and undergoing immunoprophylaxis trial in India (Kartikeyan et al., 1990).

Subunit vaccines

Failures of integral vaccines and our growing knowledge of immunoregulatory mycobacterial constituents (eg.,those causing autoimmunity and inflammatory tissue damage) have weakened the prospects of vaccines based on whole organisms. The attention has now been focussed on the development of 'subunit vaccines' based on the immunoprotective constituents of mycobacteria. Development of such simpler vaccines is based on the premise that even a single 'appropriate' antigen could provide a sufficient trigger for early activation of complete set of 'desired' immune responses to eradicate the infection. Such preparations would have many advantages over live organisms specially in immunocompromised hosts (Guleria et al., 1996). The realisation that the 'appropriate' antigens are proteins and the 'desired' responses are those mediated by T cells, precisely the Th1 population, (Kaufmann, 1993) led to the characterisation of many mycobacterial proteins (Section 1.2.6). Some such proteins have been tested for their protective efficacies as described below.

Heat shock proteins

The recombinant 65 kDa and 10 kDa hsps of M.leprae showed protection in mice against foot pad challenge with M.leprae whereas the recombinant 18 kDa hsp was ineffective (Gelber et al., 1994). In a separate study, the recombinant M.leprae 14/16 kDa low molecular weight hsp (belonging to the α-crystallin family) also did not impart protection (Mathews et al., 1985). In a recent study, a DNA vaccine based on 65 kDa hsp has been shown to afford protection in mice equivalent to that obtained with live BCG ; though immunisation with the protein itself was ineffective (Tascon et al., 1996).

Membrane proteins

Vaccination of mice with purified 22 kDa and purified or recombinant 35 kDa peripheral membrane proteins of M.leprae could not provide protection against foot pad challenge with M.leprae (Gelber et al., 1994).

Table 1.1 Reasons attributed for the variability in efficacy of the bacille Calmette - Guerin (BCG) vaccine against tuberculosis

Vaccine- related factors

- BCG strain
- Vaccine viability
- Dose
- Route of administration
- Boosting

Vaccine-recipient-related factors

- Nutrition
- Intercurrent infections
- Genetic factors : human leukocyte antigen phenotype, nramp
- Exposure to environmental mycobacteria

Secreted proteins

A DNA vaccine based on 30 kDa (antigen 85) secretory protein has shown protection comparable to BCG (Huygen et al., 1996). Another potential candidate being evaluated as a single or a multiple component vaccine is the low molecular weight secretory protein ESAT-6 of M.tuberculosis(Sorensen et al., 1995).

Other proteins

The native 28 kDa superoxide-dismutase (Gelber et al., 1994) and a DNA vaccine based on 'proline rich' 36 kDa antigen of M.leprae (Tascon et al., 1996) have not shown impressive protection in mice.

Future vaccines

Identification of the immunological mechanisms required for protection and the antigens recognised by these protective responses would provide a variety of opportunities for the development of new and possibly more effective vaccines against mycobacterial diseases.

One approach would be the stable introduction and expression of appropriate protective antigens of M.tuberculosis in suitable vectors. Following this strategy, a recombinant M.vaccae expressing the 19 kDa antigen of M.tuberculosis was prepared, though it failed to protect mice (Abou-Zeid et al., 1997). BCG is currently being pursued as a vehicle for immunodominant proteins of several pathogens (Stover et al., 1991). Other vehicles which may be used as a vector are E. coli, Salmonella typhimurium and S.typhi (European Commission, 1996).

Another rational strategy would be to identify the genes of M.tuberculosis required for virulence and then delete them, thereby creating new attenuated vaccine strains.

Due to the increased prevalence of HIV disease, there is a concern over the use of live vaccines in potentially immunocompromised recipients. One approach to this problem would be to use auxotrophs i.e., mutants that lack one or more enzymes

essential for continued growth of the organism. An ideal auxotrophic vaccine might be a double mutant in which the probability of reversion to fully viable organism is statistically remote; and that is capable of surviving for 1 to 3 months in the host before being lost. During that time, this ideal vaccine would target the appropriate antigen-presenting cells and lymphoid tissues and thus immunize the host before the auxotrophic mutation rendered it non-viable. Some auxotrophic strains of BCG have already been developed and initial results show that protective efficacy was not lost by mutation (Guleria et al., 1996).

CHAPTER II

FRACTIONATION AND CHARACTERISATION OF BCG MEMBRANE PROTEINS

2.1 INTRODUCTION

Proteins from intracellular pathogens such as mycobacteria are the targets for T-lymphocytes, the key determinants in driving the outcome of an infection towards a pathogenic or protective sequelae (Kaufmann, 1993). This realization has led to an extensive characterisation of mycobacterial proteins in order to identify candidates which could trigger desired (protective) immune responses in the host. Till date the well characterised mycobacterial proteins belong mostly to the cell wall, cytosol and culture filtrates (Young et al, 1992; Thole et al., 1995). Considerably lesser efforts have gone into the characterisation of mycobacterial membrane proteins and their interactions with the host, although the immunoreactivity of membrane constituents of gram positive and gram negative bacteria was well established (Osborn et al., 1980; Rogers et al., 1980).

Emerging evidences from several other intracellular pathogens viz., T.pallidum, Leishmania and P.falciparum have also emphasized their high immunogenicity and prophylactic potentials (Akins et al., 1993; Murray et al., 1989; Smythe et al., 1988). The major obstacle towards study of mycobacterial membrane proteins was difficulty in subcellular fractionation and resolution of membrane proteins from the underlying immunosupressive membranous glycophospholipids (Chatterjee et al., 1992; Moreno et al., 1988; Sibley et al., 1990), the lipoarabinomannan (LAM), lipomannan (LM) and phosphatidylinositol mannosides (PIM).

Using extensive detergent phase separation the previous workers could fractionate and purify only the water soluble ('peripheral') membrane proteins of Mycobacterium tuberculosis and M.leprae (Hunter et. al., 1990; Pessolani et al., 1994; Lee et al., 1992). Both these slow growing mycobacteria contained copious amount of LAM and its co-partitioning into the detergent phase posed a major obstacle for further resolution of detergent soluble ('integral') membrane proteins. This limited our knowledge about their functional and immunological relevance. However, a recent study from our laboratory (Mehrotra et al., 1995) using a fast growing mycobacterium

(M.fortuitum) describes successfully the fractionation and distinct resolution of both water and detergent soluble membrane proteins. The water solubility of LAM in this mycobacterial species was the most important consideration for its selection. This made it possible to study the detergent soluble proteins and reveal for the first time their serological distinction and immunodominance for human T cells.

This chapter describes the subcellular fractionation of BCG, and isolation and characterisation of its plasma membrane. Precise fractionation of 'integral' and 'peripheral' membrane proteins was carried out using Triton X-114 phase separation technique. The profile of these two classes of membrane proteins and partitioning of lipoarabinomannan have been characterised by SDS-PAGE.

2.2 MATERIALS AND METHODS

All chemicals and reagents used were of analytical grade obtained from B.D.H., E. merck and Ranbaxy India Ltd. Fine chemicals and biochemicals were procured from Sigma Co., USA. All reagents were prepared in triple distilled water (TDW) and autoclaved (wherever required) at 15 lb for 20 min. Glasswares were made sterile in dry heat (120^{0}C for 2 h).

2.2.1 Cultivation of mycobacteria

Requirements

Bacillus Calmette Guerin (BCG) : BCG (Danish strain) was supplied as freeze dried powder (in ampules) by BCG vaccine laboratory, Madras, India.

Lowenstein-Jensen's (LJ) medium

Malachite green : 10% (w/v) solution, prepared by dissolving the crystals in few drops of absolute alcohol and making up the final volume with TDW.

Mineral salt solution : It consisted of L-asparagine, 0.6% (w/v); KH_2PO_4, 0.4% (w/v); $MgSO_4$, 0.04% (w/v); Mg-citrate, 0.1% (w/v); glycerol, 2% (v/v) in TDW. The solution was aliquoted in 50 ml volumes and autoclaved.

Eggs : 100 ml (2 eggs) per 50 ml mineral salt solution. Eggs were washed with soap and rinsed with 70% alcohol.

Two egg masses, 50 ml mineral salt solution and 4 ml malachite green solution were stirred for 20 min in a sterile beaker. The mixture was filtered through a sterile stainless steel guaze and dispensed (~7ml) in sterile screw capped glass vials. This entire procedure was performed in a culture hood. The vials were kept in a slanting position in a container and inspissated (kept in moist heat about 70°C in a steam autoclave) for 30 min which led to the solidification of media. The vials were incubated for 48 h at 37°C in an incubator to check for contamination and then stored at 4°C till used (within 1-2 months).

Sauton's liquid medium (Sauton, 1912) : L-asparagine, 4.0g; citric acid 2.0g; dipotassium hydrogen-phosphate (K_2HPO_4), 0.5g; magnesium sulfate ($MgS0_4.7H_2O$), 0.5g; glycerol, 35 ml and Tween 80, 0.2 ml were dissolved in TDW, pH adjusted to 7.2 with 40% (w/v) NaOH solution and final volume made upto 1000 ml with TDW. The medium was dispensed (200 ml each) in 500 ml volume conical flasks, plugged with cotton and autoclaved.

Normal Saline : 150 mM NaCl in TDW.

Procedure

Freeze dried BCG, suspended in normal saline (50 µl per ampule) was used to inoculate 4-5 tubes of LJ media. The tubes were incubated at 37°C. Colonies which appeared in about 3-4 weeks were then used to inoculate fresh L-J medium for maintaining seed cultures, and Sauton's liquid medium for bulk growth (either staitonary or on an orbital shaker). All tubes and flasks were kept at 37°C. The seed cultures in LJ medium were stored at 4°C whereas the cultures from liquid medium were harvested in the late log phase of growth (~ 3 weeks) by centrifuging at 10,000 rpm for 20 min at 4°C in a Sorvall RC5C centrifuge. Bulk cultures were thus obtained by repeated passage in liquid medium. The har vested wet bacterial pellet was washed

twice with normal saline by centrifuging as above, weighed and stored at 20°C till used.

2.2.2 Acid fast staining of mycobacteria (Lartigue and Fite, 1962)

Requirements

Zeihl-Neelsen's stain

Carbol fuchsin : Basic fuchsin, 1% (w/v) and phenol, 5% (w/v) were dissolved in 10 ml of 95% alcohol by gentle warming and final volume made upto 100 ml with TDW. The stain was filtered and stored at room temperature.

Acid-alcohol : 3% (v/v) hydrochloric acid (HCl) in ethyl alcohol.

Methylene blue : 0.5% (w/v) solution in TDW.

Procedure

A droplet of cell suspension was spread as a thin film on a slide, air dried and fixed (70°C for 2-3 min on a hot plate). Carbol fuschin solution was poured on slide and the slide was gently heated from below till fumes appeared. After 10 min, it was washed with water and then twice destained with acid alcohol for 10-20 seconds. The slide was then washed under tap water and counter stained for 2-3 minutes with methylene blue reagent for non-specific staining. After washing with water the slide was air dried and observed under microscope using oil immersion lens (100 X) for visualising the acid fast mycobacteria.

2.2.3 Isolation and characterisation of plasma membrane

Requirements

Sonication buffer : 50 mM Tris-HCl, 10 mM $MgCl_2$ (pH 7.4) containing sodium azide (0.02%, w/v) and protease inhibitors (1 mM EGTA and 1 mM PMSF).

Tris buffered saline (TBS) : 10 mM Tris-HCl, 150 mM NaCl, pH 7.4.

Phosphate buffered saline (PBS) : 10 mM phosphate buffer 150 mM NaCl, pH 7.4.

Uranyl acetate solution : 1% (w/v) uranyl acetate in TDW.

Procedure

The mycobacterial cell pellet was suspended (0.2 g/ml) in sonication buffer and sonicated in an ice bath for 15 min (excluding the rest periods) using a ultrasonic processor (Heat Systems, USA, Model W-220 F). The time of sonication was standardised by monitoring the extent of cell disruption by acid fast staining of the sonicate (section 2.2.2). The sonicate was centrifuged at 20,000 g for 20 min at 4°C (on a Sorvall RC5C centrifuge using fixed angle rotor SS-34) to remove the cell wall pellet which was then suspended in TBS. The resulting supernatant was ultracentrifuged at 150,000 g for 90 min at 4°C (in a Beckman L8-M ultracentrifuge using fixed angle rotor) to yield the plasma membrane and cytosol. The membrane pellet was suspended in sonication buffer and washed twice by centrifuging as above to remove the cytosolic contaminants and finally suspended in TBS after protein estimation (as described in section 2.2.5) at a final concentration of 10-12 mg protein/ml. All fractions were stored at -20°C.

For use in tissue culture experiments a portion of bacterial mass was suspended in PBS and sonicated as above to yield cell wall, plasma membrane & cytosol. The plasma membrane was reconstituted in PBS and aliquoted (1mg protein/ml). These and cytosol aliquots (1 mg/ml) were autoclaved and stored at -20°C.

For morphological characterisation, an aliquot of the membrane was negatively stained with uranyl acetate and vesicles were visualized under the electron microscope according to the recommended protocol (Asano et al., 1973).

2.2.4 Isolation of integral (IMP) and peripheral (PMP) membrane proteins using phase partitioning (Bordier, 1981)

Requirements

Tris buffered saline (TBS): 10 mM Tris-HCl, 150 mM NaCl; pH 7.4. TBS with protease inhibitor(1 mM EGTA and 1 mM PMSF) and 0.02% sodium azide was prepared separately.

Precondensed Triton X-114 (TX-114): 10 ml (10 gm) of TX-114 containing 8

mg of butylated hydroxy toluene (antioxidant) was mixed with 490 ml of TBS and kept at 4°C for 1 h with intermittent shaking. The mixture was transferred to a separating funnel and left overnight at 30°C for separation of detergent and aqueous phases. The detergent phase (~40-50 ml) was collected, its volume again raised to 500 ml with TBS and phase separation done as above. After 3 such washings the final concentration of the detergent phase (~50 ml) collected was 20% (w/v).

Ammonium sulfate solution : 95% saturation was prepared according to table 2.1.

Acetone

Procedure

In order to ascertain the optimum concentration of TX-114 for maximum solubilisation of plasma membrane proteins, solubilisation was conducted at 3 different detergent concentrations (1%, 2% and 4%). After observing that the extent of solublisation was same with either 2% or 4% concentation, 2% detergent was routinely applied in the following manner. The membrane suspension (10 mg/ml) was divided into aliquots of 1.8 ml each and 0.2 ml of precondensed TX-114 (20%w/v) was added in each aliquot to give a final detergent concentration of 2%. Individual suspension was further diluted upto 10 ml with 2% TX-114 (in TBS, pH 7.4, containing protease inhibitors and sodium azide) and kept at 0°C for 1h with gentle intermittent shaking to get the solubilised material in a single phase. Residual insoluble material was removed by centrifugation at 150,000 g for 90 min at 4°C. The supernatant warmed at 37°C was centrifuged at 1000 g for 20 min to separate the aqueous and detergent phases. Both phases were washed three times as follows. Detergent phase (1 ml) was raised to 10 ml with TBS containing protease inhibitor and the aqueous phase (9 ml) was treated with 1 ml of 20% TX-114 and both mixtures were partitioned as described above. Proteins in the pooled detergent (integral membrane proteins, IMP) and aqueous phases (peripheral membrane proteins, PMP) were recovered by precipitation with either 5 volumes of chilled acetone (det.phase) or at 95% ammonium sulfate saturation (aq. phase) according to Table 2.1. The

Table 2.1 FINAL CONCENTRATION OF AMMONIUM SULFATE: PERCENTAGE SATURATION AT 0°

Initial concentration of ammonium sulfate (percentage saturation at 0°)	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

protein precipitate from both phases was suspended in PBS. The aqueous phase proteins (PMP) were dialysed against PBS for 72 hrs and stored at -20°C. An aliquot of each fraction was autoclaved before storage for use in tissue culture experiments.

2.2.5 Protein estimation (Markwell et al., 1978)

Requirements

Solution A : 2% Na_2CO_3 in NaOH and 1% SDS. Stored at room temperature.

Solution B : 0.5% $CuSO_4$. $5H_2O$ in 1% sodium citrate. Stored at room temperature.

Solution C : Prepared fresh by mixing 1 ml of solution B to 50 ml of solution A.

Solution D : Folin-Ciocalteu reagent (1x) prepared fresh by diluting the stock with TDW.

Standard protein solution : 1 mg/ml Bovine serum albumin (BSA) solution prepared in TDW. Stored at 4°C.

Procedure

A standard curve (Fig. 2.1) was plotted taking serial concentrations of BSA (10-80 µg/0.1 ml TDW) in duplicates. A blank tube was prepared by taking 0.1 ml of TDW. Different dilutions of unknown sample were taken in a final 0.1 ml TDW. To each tube 3 ml of solution C was added and vortexed. After keeping the tubes for 10 min at room temperature 300 µl of solution D was added and vortexed throughly. The tubes were incubated at 37°C for 30 min and absorbance measured at 750 nm using a Shimadzu (UV 240) spectrophotometer.

2.2.6 SDS-polyacrylamide gel eletrophoresis (SDS-PAGE) (Laemmli, 1970)

Requirements

Resolving gel buffer : 1.5 M Tris-HCl, pH 8.8.

Stacking gel buffer : 0.5 M Tris-HCl, pH 6.8.

Electrode buffer : 0.3 Tris-HCl, 1.4 g Glycine, 0.1 g SDS in 100 ml TDW.

Acrylamide stock : (30% T, 2.7% C) 29.2 g acrylamide and 0.8 g bisacrylamide in 100 ml TDW. Filtered and stored in dark bottle.

Ammonium per sulfate solution (APS) : 10% (w/v) in TDW.

N,N,N,N-tetra methyl ethylene diamine (TEMED)

Sample loading buffer : 0.5 M Tris-HCl (pH 6.8), 6 ml; 10% SDS, 10 ml; glycerol, 5 ml; 0.5% brormophenol blue, 2.5 ml; TDW, 26.5 ml. Before use 50 µl of ß-mercaptoethanol was added to 950 µl of sample loading buffer. A desired volume of this was used with protein samples.

Agarose : 1% (w/v) in TDW.

Procedure

SDS-PAGE was performed in minislab gel apparatus (Broviga, India). Plates were sealed with 1% agarose solution and resolving gels (7 cm, 0.1 mm) of desired percentage were cast according to the table 2.2. After polymerisation 4% stacking gel (2 cm) was poured. Protein samples of appropriate concentration were diluted (1:2) with sample loading buffer and loaded into the wells. Gels were run in electrode buffer at a constant current of 15 mA for 1.5 to 2 h till the bromophenol blue dye reached near the end. The gels were stained with either coomassie blue or silver stain as described below.

2.2.7 Staining of gels

Requirements

Coomassie blue : 0.5 g coomassie brilliant blue solution in 40% methanol, 10% acetic acid.

Destaining solution : 40% methanol, 10% acetic acid in TDW

Silver nitrate reagent (for protein staining) : A 20% (w/v) silver nitrate solution was prepared freshly and added dropwise with stirring to ammoniacal sodium hydroxide solution (0.36% NaOH, 21 ml and 12.5% ammonia solution, 1.4 ml) with

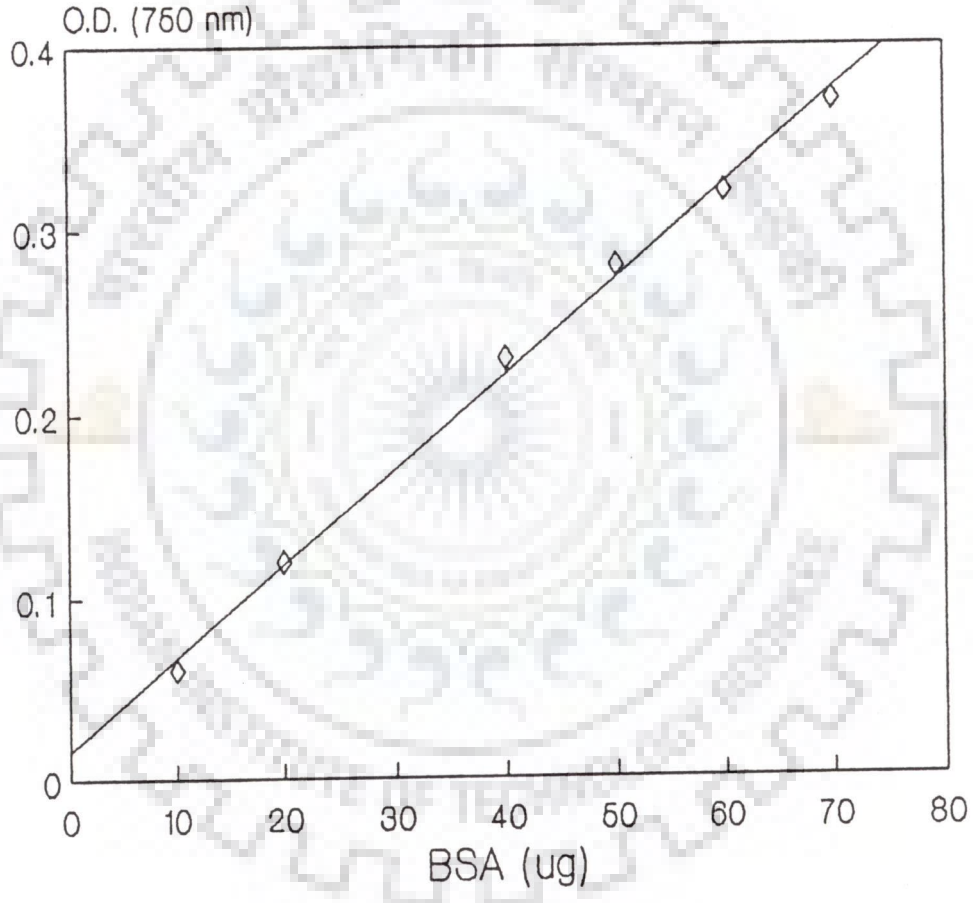

Fig. 2.1 Standard curve for protein estimation.

Table 2.2 SDS-PAGE Gel Preparation

%T (acrylamide monomer)	ddi H_2O (ml)	Gel buffer solution* (ml)	Acrylamide / bis solution 30% stock (37.5:1) (ml)
4%	6.15	2.50	1.33
5%	5.80	2.50	1.67
6%	5.45	2.50	2.00
7%	5.15	2.50	2.33
8%	4.80	2.50	2.67
9%	4.45	2.50	3.00
10%	4.15	2.50	3.33
11%	3.80	2.50	3.67
12%	3.45	2.50	4.00
13%	3.15	2.50	4.33
14%	2.80	2.50	4.67
15%	2.45	2.50	5.00
16%	2.15	2.50	5.33
17%	1.80	2.50	5.67

*Resolving Gel buffer - 1.5 M Tris-HCl, pH 8.8
*Stacking Gel buffer - 0.5 M Tris/HCl, pH 6.8

	Catalyst	
	10% APS*	TEMED*
Analytical Resolving Gel	50 µl	5 µl
Stacking gel	50 µl	10 µl
Preparative Resolving Gel	25 µl	2.5 µl
Stacking Gel	50 µl	10 µl

**Note: Amounts are per 10 ml gel volume. Different amounts of catalyst are added for analytical and preparative gels. To make 10% APS, dissolve 100 mg in 1 ml of deionized water.

constant stirring till the appearance of a brown precipitate. The final volume of the solution was raised to 100 ml with TDW.

Silver nitrate reagent (for LAM staining) : A silver nitrate solution described as above was added to ammoniacal sodium hydroxide solution (0.36% NaOH, 28 ml and 25% ammonia solution, 2 ml).

Periodic acid solution : 0.7% (w/v) in 40% ethanol, 5% acetic acid solution.

Developer : 2.5 ml of citric acid and 0.25 ml of 38% formaldehyde solution were added freshly to 500 ml TDW.

Procedure

Coomassie blue staining (CBS) : The gels were dipped overnight in coomassie blue stain and then for 3-4 h in destaining solution till protein bands appeared against a clear background. Gels were washed with water and stored in 5% acetic acid solution at 4°C.

Silver staining : Silver staining of proteins was done according to the established protocol (Wray et al.,1981). The gels were left overnight in 50% methanol with gentle shaking. It was then washed with TDW for 10 min with one change in between. The gels were then stained for 30 min at room temperature in silver nitrate reagent with continuous shaking and then washed twice with TDW and soaked in the developer till the appearance of bands of desired intensity.

For preferential staining of lipoglycans such as LAM (lipoarabino-mannan), LM (lipomannan) and PIM (phosphatidylinositol mannosides), a modified protocol was followed (Tsai and Frasch, 1982). The gels were soaked overnight in 40% ethanol, 5% acetic acid solution. They were then placed for 5-7 min in periodic acid solution with gentle agitation, washed three times (15 min each) with TDW and then stained with silver nitrate reagent for 15 min. Again the gels were washed three times with TDW as above and then soaked in the developer. The dark brown coloured band corresponding to LAM, LM and PIM appeared within a few minutes before the

appearance of protein bands. Immediately the gels were washed thoroughly with TDW and stored at 4°C in TDW.

2.2.8 Determination of molecular mass of proteins

Standard marker proteins (for SDS-PAGE) of known molecular masses were obtained from Sigma, USA. The mixture was reconstituted in TDW to give a stock protein concentration of 2 mg/ml. This stock was diluted (1:1) with sample loading buffer, boiled for 5-10 min and loaded (20-25 µg protein/well for CBS and 10-15 µg/well for silver staining). The unknown protein samples were diluted similarly and loaded in adjacent wells. After electrophoresis and staining, the Rf values of each marker band and unknown sample was calculated by taking the ratio of the distance travelled by individual bands to the distance travelled by the dye. A curve of log molecular mass of standard marker and the respective Rfs was plotted (Fig. 2.2). With the Rf value of unknown samples their molecular mass could be calculated from this plot.

2.3 RESULTS

2.3.1 Yield of BCG

Bulk cultures in Sauton's media under stationary condition yielded at an average 1 g wet bacterial mass per litre media. This yield increased 3-4 folds when the cultures were kept on an orbital shaker. The property of mycobacteria to retain the Zeihl-Neelsen stain even when treated with mineral acid was used to assess the purity and homogeneity of cultures. BCG appeared as bright pink rods when seen under the microscope (100 x magnification).

2.3.2 Subcellular fractionation, yield and characterisation of plasma membrane

Sonication of BCG upto 15 min led to the breakage of 60-70% of cells as monitored by acid fast staining before and after sonication. The broken cell debris took the methylene blue stain and lost the rod shape morphology as compared to bright

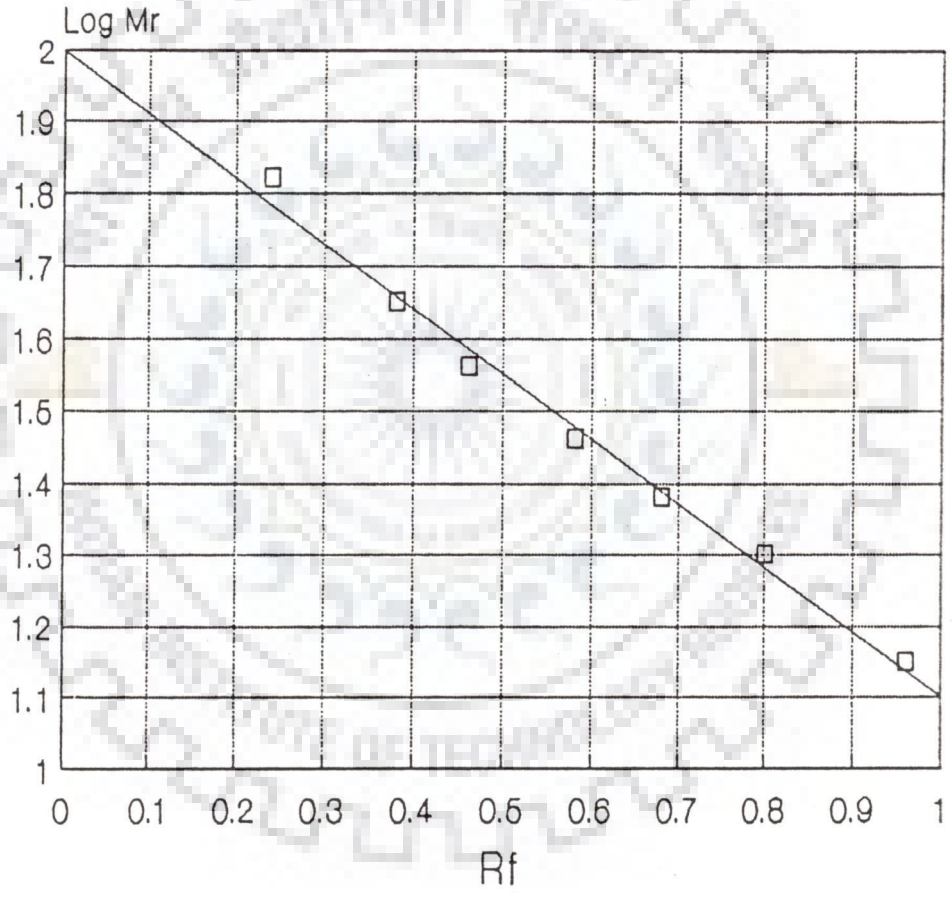

Fig. 2.2 Standard curve for molecular weight determination of proteins.

pink rod like intact cells.

The subcellular fractions viz. cell wall, cytosol and plasma membrane were isolated by differential centrifugation. Washing of the plasma membrane pellet by suspending in sonication buffer and recentrifugation made it free of cytosolic contaminats. Under the electron microscope the negatively stained membrane showed characteristic vesicles of 50-100 nm diameter (Fig. 2.3). At an average each gm of wet bacterial mass yielded 6 mg membrane protein. The subcellular fractions viz.. cell wall, cytosol and plasma membrane obtained from each batch of cultured BCG were checked for protein profiles on SDS-PAGE. Each batch gave reproducible protein profile. The protein profiles of cell wall, cytosol and plasma membrane shown in Fig. 2.4, very clearly demonstrate the distinction between cytosol and plasma membrane as evident from the major bands in the two fractions. However, there was considerable overlap in the major bands of cell wall and membrane.

2.3.3 Yield and characterisation of integral (IMP) and peripheral (PMP) membrane proteins

Plasma membrane was extracted with 2% Triton X-114 at a detergent to protein ratio of 10:1 (w/w) and the solubilized material was phase partitioned. Approximately 50% of the plasma membrane remained insoluble. The final protein recovery, after washing and precipitation, was as follows (average of 9 batches) :

0.06 mg of IMP/mg of membrane (0.4 mg/g of wet bacterial mass).

0.045 mg of PMP/mg of membrane (0.3 mg/g of wet bacterial mass).

SDS-PAGE revealed distinct and batch to batch reproducible protein profiles of the two phases (Fig. 2.4). The integral protein pool showed four major bands of 20, 25, 40 and 68 kDa, whereas the peripheral protein pool showed six major bands of about 16, 21, 30, 34, 54, 66 kDa. Almost all major proteins of the membrane could be located in either of the two phases.

2.3.4 Partitioning of LAM, LM and PIM

Staining of LAM and its degradation products, LM and PIM, in membrane and its subcellular fractions is shown in Fig. 2.5. Typical diffused zones of LAM, LM and PIM as seen in M.leprae cytosol also were distinctly stainable in BCG membrane and in the detergent soluble integral protein fraction. Whereas, only PIM region was stained in the water soluble peripheral protein fraction.

2.4 DISCUSSION

The key to successful purification of proteins from a particular compartment of an organelle lies mainly in its initial subcellular fractionation. Mycobacteria are tough organisms with most of them being fairly resistant to enzymatic digestion and detergent lysis. Typically, they have been broken by mechanical stress (sonication, grinding with abrasives or shearing in the french pressure cell) and then fractionated by differential or density gradient centrifugation. Technique for the isolation and characterisation of mycobacterial plasma membranes was worked out by Brodie and his group (Brodie et al., 1979) which was mainly done on fast growing mycobacteria but could potentially be applied to the slow growers too. In the present study we have used this well established protocol for the subcellular fractionation and isolation of plasma membranes of BCG. Previous reports on membrane preparation of Mycobacterium leprae (Hunter et al., 1990) and M.tuberculosis (Lee et al., 1992) have adopted similar protocol with slight modifications. Our yield of the BCG plasma membrane (which is relative to wet bacterial mass) could not be compared with that reported for M.leprae (Hunter et al., 1990) which is relative to dry weight of bacteria. Protein profile of the plasma membrane (Fig. 2.4) was fairly distinct from that of cytosol indicating the clarity of preparation. The overlap in the major bands of cell wall and membrane is expected because cell wall pellet contains unbroken cells and contaminating membrane which even after sonication remain attached to the walls but contamination of membrane with the cell wall is ruled out.

Enzymatic (using membrane marker enzymes) and morphological (by electron

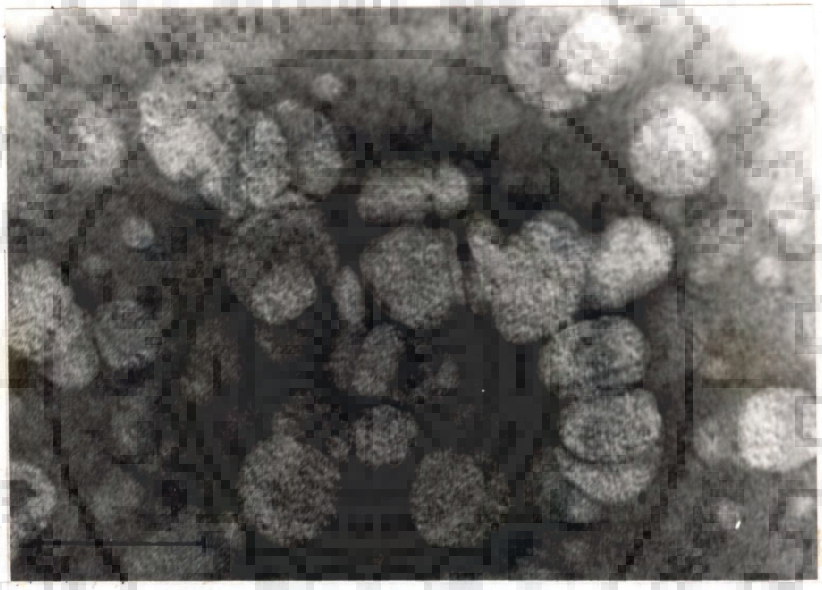

Fig. 2.3 Electron micrograph of negatively stained membrane vesicles (mag. X 170,000). Bar , 100 nm.

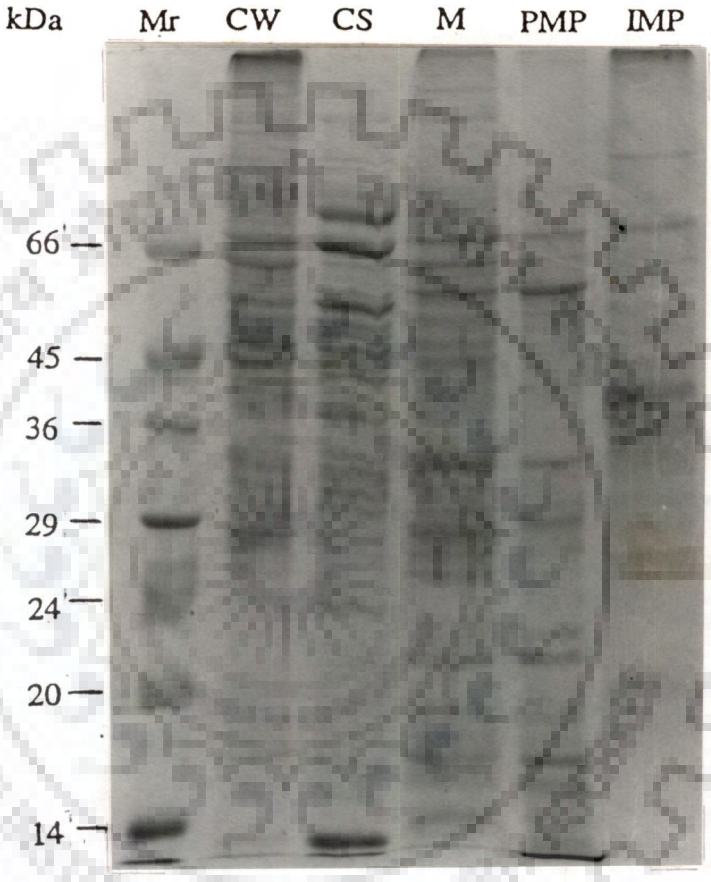

Fig. 2.4 SDS - PAGE (12% gel) showing protein profile of subcellular fractions of BCG after coomassie blue staining. Cell wall (CW), cytosol (CS), membrane (M), peripheral and integral membrane proteins (PMP and IMP).

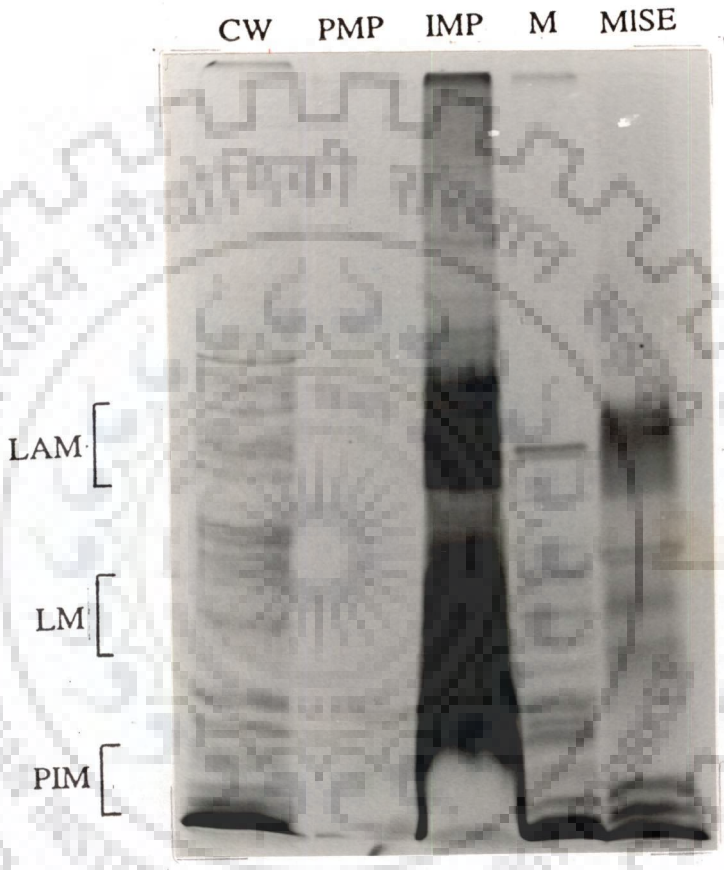

Fig. 2.5 SDS - PAGE with preferential staining for lipoarabinomannan (LAM), lipomannan (LM) and phosphatidylinositol mannosides (PIM) in subcellular fractions of BCG. Lanes : CW, cell wall; PMP, peripheral membrane proteins; IMP,integral membrane proteins; M, membrane. M. leprae cytosol (MISE) was used as a positive control.

microscopy) characterisation are used to assess the purity and homogeneity of the isolated membrane preparations (Asano et al., 1973). We tried to determine the specific activity of a membrane marker enzyme (ATPase) in plasma membrane and cytosol but could not find appreciable difference in enzyme activities in these two compartments as the values in cytosol were too high which possibly was due to leaching of this losely bound protein into the cytosol during sonication. We therefore, relied firstly, on morphological characterisation of plasma membrane which upon negative staining under electron microscopy showed characteristic vesicles (Fig. 2.3) and secondly, on the distinct protein profiles of plasma membrane and cytosol (Fig. 2.4).

Phenol biphasic solvent has been successfully used to resolve the O-antigenic lipopolysaccharides (LPS) from outer membrane proteins of gram-negative bacteria (Hunter et al., 1990). However, the approach did not work for mycobacterial membranes because the proteins get distributed in both (LAM containing) phenol phase and the aqueous phase. Later the detergent phase separation protocol of Bordier (1981) could be successfully applied to resolve the membrane proteins of M.leprae (Hunter et al., 1990) and M.tuberculosis (Lee et al., 1992). The temperature dependent phase separation property of the non-ionic detergent TX-114 has been expeditiously utilized by Bordier (1981) to exclusively seperate the hydrophilic water soluble and amphiphillic detergent soluble proteins. At 0°C TX-114 remains as a homogeneous solution but separates into distinct aqueous and detergent phases above 20°C. The simplicity of the methodology, less time requirement , reproducibility of the partitioning pattern of proteins/polypeptides and retention of biological activity due to mild nature of the detergent make it a valuable technique for membrane research. It can be applied to micro as well as preparative and even industrial scale.

Despite the successful fractionation of water soluble (peripheral) and detergent soluble (integral) membrane proteins of both M.leprae and M.tuberculosis a further analysis of integral membrane protein pool was not undertaken in view of its heavy

contamination with immunosuppressive LAM, thereby limiting our knowledge about their functional and immunological relevance. Recently, a study from our laboratory using a fast growing mycobacterium M.fortuitum as a model delineated the immunodominance of the detergent soluble integral membrane proteins for human T-cells (Mehrotra et al., 1995). This mycobacterium contained significantly lesser amounts of LAM as compared to M.leprae and M.tuberculosis which partitioned exclusively into the aqueous phase enabling easy removal and a comparative immunological evaluation of both classes of membrane proteins.

In the present study using the biphasic TX-114 extraction we could separate the integral and peripheral membrane proteins of BCG with great precision like in the case of M.fortuitum as is apparent from SDS-PAGE results (Fig. 2.4). All major proteins of the membrane could be located in either of the two phases. As we had observed in M.fortuitum the initial solubilisation of the plasma membrane of BCG in the detergent was also only 30-40% which affected the final yields of integral and peripheral proteins. This solubilisation could not be increased further even at higher concentration of the detergent. Moreover, it is very difficult to reconstitute the undissolved membrane pellet and re-extract it with the detergent for a second time.

The plasma membrane, its subfractions (integral and peripheral protein pools) and cytosol of BCG were stained for the presence of LAM using M.leprae soluble extract as a positive control as it is known to contain large amounts of this immunosupressive moiety. Characteristic LAM band in 30-40 kDa region could be visualised in M.leprae soluble extract and integral membrane protein pool of BCG (Fig. 2.5) but not in cytosol and peripheral protein pool. However, both these fractions showed the presence of PIM by staining. These observations are in agreement with those of other slow growing species viz., M.leprae and M.tuberculosis. Comparative quantitative estimates of LAM in different mycobacterial species or its subcellular distribution within any species has not been determined. The features determining water or detergent solubility of membrane LAM, of "fast" and "slow" growing mycobacteria respectively, are also not fully understood, though these properties may have a bearing on the physiological role of LAM.

CHAPTER III

IMMUNOLOGICAL ANALYSIS OF INTEGRAL AND PERIPHERAL MEMBRANE PROTEINS

3.1 INTRODUCTION

The highly immunogenic membrane proteins described from various pathogens [T.pallidum (Akins et al., 1993), Leishmania (Murray et al.,1989) and P.falciparum (Smythe et al., 1988)] belong to the detergent soluble "intergral" membrane protein pool. However as discussed in the previous chapter (section 2.1 and 2.4) our knowledge about the mycobacterial membranes was limited to the water soluble "peripheral" proteins of M.leprae (Hunter et al., 1990) and M. tuberculosis (Lee et al., 1992) where copartitioning of immunosuppressive LAM together with the IMPs posed a major obstacle in their characterisation and immunological evaluation. Recently, the work of Mehrotra et al.(1995) on M. fortuitum made a comparative evaluation of immunological relevance of mycobacterial membrane proteins thereby revealing the immunodominance of 'intergal' membrane proteins for human T cells.

Immunogenicity of membrane proteins, especially the "integral" ones, has been attributed to their inherent hydrophobicity which could be accentuated by a naturally occurring lipid modification (Chamberlain et al., 1989). According to the recently unravelled mechanism of processing and presentation of antigenic proteins to the T cell (Germain, 1994) interaction between major histocompatibility complex (MHC, or more pertinently, MHC class II) molecule and antigenic peptide is largely hydrophobic and broadly specific, allowing for a considerable level of promiscuity (Hammer et at., 1993). The membrane proteins could thus provide suitable ligands to a range of MHC alleles expressed in cross section of a population resulting in appropriate T cell responses. Lipid modification, on the other hand, has long been demonstrated to enhance the immunogenicity of proteins, including the mycobacterial ones (Akins et al., 1993; Deres et al., 1989; Rees et al., 1993). The recently discovered processing and presentation of lipid antigens to a specific subset of cytotoxic T cells in MHC restricted manner (Beckman et al., 1994) has given a new perspective to the role of lipids in T cell responses.

The present chapter deals with antigenic definition of the plasma membrane

proteins of BCG using polyclonal and monoclonal antibodies (moAbs). The study exemplifies how moAbs can be used not only to detect the presence of particular proteins across the species but also for their antigenic characterisation. Three previously well characterised mycobacterial proteins (19, 33/36 and 38 kDa) were found to be constituents of the BCG IMP. A comparison of membrane antigens of BCG (a slow grower) has also been made with those of M. fortuitum (a fast grower). Finally, a comparative evaluation of the human T cell activating potential of membrane and its subfractions was made.

3.2 MATERIAL AND METHODS

3.2.1 **Enzyme-linked immunosorbant assay** (ELISA) (Chaturvedi et al., 1991)

Requirements

Primary antibodies

Monoclonal antibodies : A panel of 30 antimycobacterial antibodies (Table 3.1) against shared epitopes of 17 different mycobacterial antigens (Engers et al., 1985; Engers et al., 1986; Khanolkar - Young et al., 1992) were provided by IMMLEP-IMMTUB monoclonal antibody bank of W.H.O. (Courtesy of Dr. T.M. Shinnick, CDC, Atlanta, USA). MoAbs MLO2, ML34 (Ivanyi et al., 1985) and TB78 (Coates et al., 1981) were provided by Dr. J. Ivanyi (MRC-TB and RI Unit, London, UK).

Polyclonal rabbit antisera : Polyclonal rabbit antisera was raised according to a recommended protocol (Mehrotra et al., 1995). Rabbits (1.5-2 kg) were injected intradermally (distributed at four sites on preshaven back) with approximately 300 µg (in 0.5 ml PBS) of antigens (M,IMP and PMP) emulsified in 0.5ml of Freund's incomplete adjuvant (FIA, Difco, USA). 3-5 biweekyl boosters were given in the same manner. Blood samples were collected every week after 3rd booster by an incision in the marginal ear vein and optimal titres were determined by ELISA (as described below). For separating the serum, blood samples were kept at 37°C for 1-

Table 3.1. **The used panel of monoclonal antibodies against mycobacterial antigens.**

S.No.	Antigens	Monoclonal Antibodies*: WHO and Contributor's () Code
1.	10 kDa (HSP)	mc9245 (CS 01)
2.	14/16 kDa	IT1 (F23-49-5), IT4 (F24-2-3), IT20 (WTB 68 A1)
3.	18 kDa (HSP)	mc8026 (L 5)
4.	19 kDa	IT10 (F29-47-3), IT12 (HYT 6), IT19 (WTB 23 H1), IT54 (HYT 10)
5.	20 kDa	IT38 (CITB 3H9)
6.	23/28 kDa (SOD)	mc5041 (SA1 D2D), IT61 (F116-5)
7.	25 kDa	IT52 (HBT 4)
8.	32-33 kDa (Ag85)	IT49 (HYT 27)
9.	33/36 kDa (PRA)	IT59 (F67-1)
10.	38 kDa	IT21 (HYT 28), IT47 (HBT 12), IT65 (HAT 2)
11.	40 kDa	IT7 (F29-29-7)
12.	56 kDa	IT43 (HBT3)
13.	65 kDa (HSP)	IT56 (CBA1), IT64 (HAT5), mc4243 (IIIC8), mc5205 (IIH9), (TB78)
14.	71 kDa (HSP)	IT40 (HAT 1), IT41 (HAT 3)
15.	81 (+38,27) kDa	IT35 (MH 1)
16.	85 kDa	IT45 (HBT 8), IT57 (CBA 4)
17.	LAM	mc4311 (L 1), (ML34)

*mAbs reactive only with `shared' epitopes of respective antigens were used.

`mc' denotes mAbs raised against M.leprae.

`IT' denotes mAbs raised against M.tuberculosis.

HSP, heat-shock protein; SOD, superoxide dismutase; PRA, proline rich antigen; Ag85, Antigen 85 complex; LAM, Lipoarabinomannan.

2 h, then overnight at 4°C for clot formation and centrifuged at 3000 rpm for 20 min. Antisera from all the bleeds were pooled and enriched for immunoglobulin fraction(IgG) by precipitation at room temperature with saturated ammonium sulfate solution in the ratio 40:60 (ammonium sulfate : antisera). The precipitate was pelleted at 12,000 g for 20 min and washed with a saturated ammonium sulfate solution mixed in the same ratio (40:60) with water. After centrifugation as above the immunoglobulin pellet was dissolved and dialysed in PBS at 4°C for 72 h. Again antibody titres were determined by ELISA.

Secondary antibody

Affinity purified peroxidase conjugated anti-mouse and anti-rabbit immunoglobulins (Sigma).

Tris buffered saline - Tween (TBS-T): 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 and Tween-20, 0.05%(v/v).

Coating buffer (pH 9.5) : Na_2CO_3 (0.398 g) and $NaHCO_3$ (0.933 g) in 250 ml TDW.

Substrate buffer (pH 5.0) : Citric acid (1.16 g) and Na_2HPO_4 (1.8 g) in 250 ml TDW.

Substrate : 10 mg orthophenylenediamine (OPD) in 25 ml substrate buffer containing 25 µl H_2O_2 (30%). Substrate was prepared freshly.

Stopping reagent : 7% H_2SO_4 in TDW

Procedure

ELISA was performed in 96 well round bottomed plates. Antigens diluted (100 µg protein/ml in the case of moAbs and 25 µg protein/ml in case of polyclonal antisera) in coating buffer were dispensed (50 µl/well) and kept overnight at either 4°C in a humid box (wet coating)or left uncovered at room temperature (dry coating). After washing the antigen coated wells, the nonspecific binding sites were blocked by incubating (2 h, room temperature) the plates with 1% milk powder (Anikspray) in TBS-T (100 µl/well). The appropriate dilutions (50 µl/well) of primary antibody (in

1% milk-TBS-T) were incubated (37°C, 90 min with polyclonal antisera or 4°C, overnight with moAbs) in duplicate antigen coated and buffer coated blank wells. After five washings with TBS-T, the plates were incubated (37°C, 90 min) with 1:1000 dilutions (in 1% milk-TBS-T, 50 µl/well) of peroxidase conjugated, affinity purified secondary antibody (anti-rabbit or anti-mouse immunoglobulins). Colour was developed by adding substrate (50 µl/well). After 20 min the reaction was stopped by adding 7% H_2SO_4 solution (50 µl/well) and optical densities (ODs) were read at 492 nm in a ELISA reader (Titertek Multiskan, Flow labs, USA).

3.2.2 Immunoblotting (Towbin et al., 1979)

Requirements

Transfer buffer : Glycine (192 mM); Tris-HCl (25 mM); methanol (20%) in TDW.

TBS-T : Same as used in ELISA.

Primary antibody : MoAbs and polyclonal rabbit antisera (as described in sec 3.2.1).

Secondary antibody : Same as used in ELISA.

Substrate : 4-Chloro-1-naphthol (50 mg) dissolved in methanol (17 ml) and final volume raised to 100 ml with TBS containing 70 µl H_2O_2 (30%). Substrate was prepared freshly.

Amido black stain (Sigma) : Stock was diluted 10 times with TDW and stored at 4°C.

Nitro cellulose paper : 0.45 µM pore size (Sigma).

Skimmed milk powder : (Anikspray; Lipton India Ltd.).

Procedure

The protein after SDS-PAGE (as described in section 2.2.6) were electroblotted onto nitro cellulose paper in transfer buffer at 40 V for 4 h, using a transblot apparatus (Bio Rad, USA). Individual nitro cellulose strips were blocked with 3% skimmed milk powder (in TBS-T) for 2 h at room temperature. Subsequently, incubation (2 h, room

temperature) was done with primary antibodies appropriately diluted in 1% milk-TBS-T. After washing (five times with TBS-T and lastly with TBS), bands were developed using the substrate solution. Standard molecular mass marker strips were developed separately by dipping the strips in amido black solution for 2-3 min and washing with TDW to visualise the bands.

3.2.3 T-lymphocyte proliferation assays

Requirements

Study subjects : Study subjects comprised 20 volunteers falling into categories which are normally considered as 'responders' for mycobacterial antigens (Kaufmann, 1993; Ottenhoff et al., 1989). Thus, 17 subjects were patients of tuberculoid (TT/BT) leprosy taken from the wards of skin center, base hospital, Lucknow, and the remainig 3 were healthy BCG vaccinated laboratory workers. Patients were classified according to clinico - bacteriological and histopathological criteria of Ridley and Jopling (1966) and were receiving multidrug therapy (WHO, 1988) for periods upto 4 weeks at the time of study.

Mycobacterial antigens/mitogens : Mycobacterium leprae soluble extract (MISE) derived from armadillo was provided by WHO (courtsey Dr. R.J.W. Rees, NIMR, London, UK). Purified protein derivative (PPD) of M. tuberculosis was obtained from central veterinary laboratory (Weybridge, UK). Phytohaemagglutinin (PHA), a T cell mitogen was obtained from Sigma, USA. Plasma membrane and subfractions were prepared from BCG (as described in section 2.2.4). M. leprae soluble extract, PHA and PPD were sterilized by passing through 0.2 µM syringe filter whereas BCG membrane and its subfractions were sterilized by autoclaving at 15 lbs for 15 min.

Ficoll-Isopaque (Sp. gr. 1.077) : was obtained from Sigma, USA.

RPMI 1640 (Sigma) : RPMI powder (pack for 1 litre media); 25 mM (6 g) HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) or (6.5 g) HEPES Na Salt; 26.7 ml of 7.5% $NaHCO_3$ were dissolved in ~800 ml TDW. To it, 100 µl (1 mg)

amphotericin B(Sigma) and 1 ml of 50 mg/ml gentamycin (Sigma) were added. The pH (7.4) was adjusted with 1N-NaOH or 1N-HCl and final volume made upto 1 litre with TDW. The media was sterile filtered and stored in 100 ml aliquots in autoclaved bottles at 4°C.

Pooled normal serum (PNS) : 20 ml blood each was taken from 4-5 healthy laboratory volunteers in sterile centrifuge tubes and kept at 37°C for 1-2 hrs, then overnight at 4°C to separate the serum. The contents of the tubes were centrifuged at 3000 rpm for 30 min and serum was separated with the help of sterile pasteur pipette and pooled. Pooled serum was heat inactivated at 56°C for 1h (to inactivate the complement), aliquoted (5 ml each) in sterile glass vials and stored at -20°C.

Scintillation fluid : 5 g PPO (2, 5, diphenylene oxazole) and 0.1 g POPOP (2, 2'-p- phenylene bis 5-phenyloxazole) were dissolved in 1 litre of sulfur free toluene and stored in dark bottles at room temperature.

^{3}H-thymidine : (BARC, Bombay, India) Sp. activity, 17 Ci/m mol.

Heparin (powder) : Reconstituted (10 mg/ml (~1000 U/ml); in PBS).

Procedure

Isolation of mononuclear cells (Boyum, 1968)

10 ml heparinised blood (10 U heparin/ml blood) taken from study subjects was centrifuged at 2000 rpm for 10 min at room temperature to separate plasma. The cell pellet was reconstituted in about 10 ml of heparinized RPMI (10 U/ml) by gentle mixing. 5 ml each of cell suspension was layered into tubes containing 3 ml ficoll and centrifuged at 2000 rpm for 20 min at 20-22°C to separate the mononuclear cells. Medium above the monocyte layer (white) containing most of the platelets was gently aspirated using a sterile pasteur pipette and discarded very carefully, the monocyte layer (~3ml) was collected by aspiration in a centrifuge tube and dilued with 3 volumes of heparinized RPMI. The tubes were centrifuged at 1500 rpm for 15 min at 8-10°C. The resultant pellet was resuspended in plain RPMI (~6ml) and washed by centrifuging

at 1200 rpm for 10 min. This was repeated thrice. Finally, the peripheral blood mononuclear cell (PBMC) pellet was suspended in (2-3 ml) RPMI containing 10% PNS and counted in a haemocytometer by diluting an aliquot (20 µl) 5 times in RPMI. The PBMC was suspended at a final concentration of $2x10^6$ cells per ml in RPMI containing 10% PNS to be used in cultures as described below.

Setting of cultures : PBMC ($2x10^6$ cells/ml) were dispensed (0.1 ml/well) in 96 well flat bottomed culture plates (Corning, USA) cultures were set in triplicates with or without the indicated antigen/mitogen concentrations (10 µl/well), supplemented with 10% heat inactivated pooled normal serum (final culture volume = 0.2 ml/well) and incubated (37°C, 5% CO2, 95% humidity) in a CO_2 incubator (Jouan, France). After pulsing (1 µCi/10 µl per well) with ^{3}H-thymidine for the last 18 hrs of the incubation period, the cells were harvested using a semiautomatic cell harvester (Cambridge Technology, USA) and radioactivities were counted in a beta counter (LKB, Sweden). Culture period was 6 days.

Statistical significance of results was assessed by paired t test. $P < 0.05$ was considered significant.

3.3 RESULTS

3.3.1. Antigenic characterisation of BCG membrane proteins

Using polyclonal antisera

The antigenic distinction between IMP and PMP pools was ascertained by ELISA and immunoblotting using polyclonal antisera raised in rabbits.

Antibody titres of all the three antisera (anti-M, anti-IMP and anti-PMP) were comparable, giving an OD of ≥3 at 1:1000 or 1:5000 dilutions when tested by ELISA using corresponding antigens (Fig.3.1a). The cross reactivities of IMP with anti-PMP and PMP with anti-IMP antisera were lowest at 1:125,000 dilution (Fig.3.1b). Some cross-reaction (higher in case of IMP with anti-PMP) was evident at 1:5000 and 1:25,000 dilutions though the differences between ODs (homologous and heterologous pairs) were high (Fig.3.1b). However, in immunoblotting (which is a less

sensitive technique than ELISA) antisera used at 1:1000 dilution produced a satisfactory discrimination while highlighting the major serologically active bands of both IMP and PMP pools (Fig. 3.2). Homologous reaction revealed an intense band of ~30 kDa mol wt and several minor bands above 40 kDa region within IMP pool, some of which overlapped with the (35-40 kDa) diffusely stained lipoarabinomannan (LAM). PMP showed a major band at ~36 kDa and few other intense bands condensed in the region of 60-66 kDa. The serological cross reactivity between IMP and PMP was almost negligible. Two faint bands could be seen around 66 kDa region, when IMP was cross reacted with anti-PMP antiserum.

In order to check the extent of cross reactivity between 'slow grower' BCG and 'fast grower' M.fortuitun, the IMP pool of both species were cross reacted with polyclonal antisera (IMP and anti-IMP antiserum of M. fortuitum were available in the laboratory). Fig.3.3 shows that the two species had very little cross reactivity. Even the profile of serologically dominant bands was quite distinct, with BCG-IMP showing only one major band whereas M. fortuitum-IMP showed 5 major bands. Previous studies in our laboratory have already shown that M. fortuitum contains appreciably less amount of LAM which is entirely water soluble. This observation is very clearly brought out in Fig. 3.3 where we see a characteristically diffused staining of LAM in BCG-IMP, spreading from 40 to 60 kDa region. No such smear is seen in M.fortuitum-IMP.

Using monoclonal antibodies

A battery of 32 moAbs against shared epitopes of 17 mycobacterial antigens, including LAM (Table 3.1), were used in ELISA to detect the presence of previously known molecules in BCG membrane or its subfractions. The results are depicted in Fig. 3.4 which once again underscore the distinction between IMP and PMP. The 19, 33 and 38 kDa proteins were major constituents of IMP (OD= 0.11, 0.14 and 0.13 respectively) whereas 14/16 kDa protein (OD= 0.18) was present in PMP. In addition, the 40 kDa protein partitioned in both the fractions (OD= 0.15 in IMP and 0.47 in PMP). The 65 kDa (HSP) and 85 kDa proteins were detected marginally (OD 0.1 to

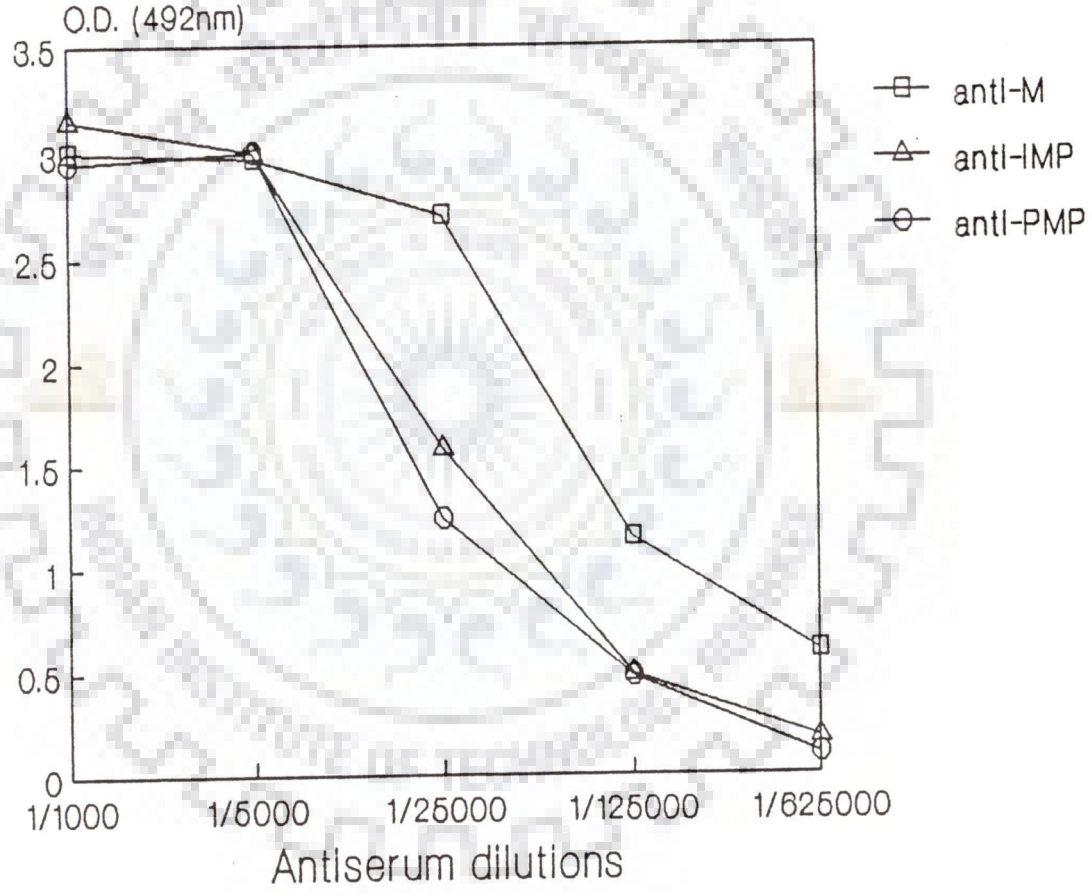

Fig 3.1(a) Antibody titres of BCG membrane and its subfractions raised in rabbits. O.D. values as dsetermined by ELISA.

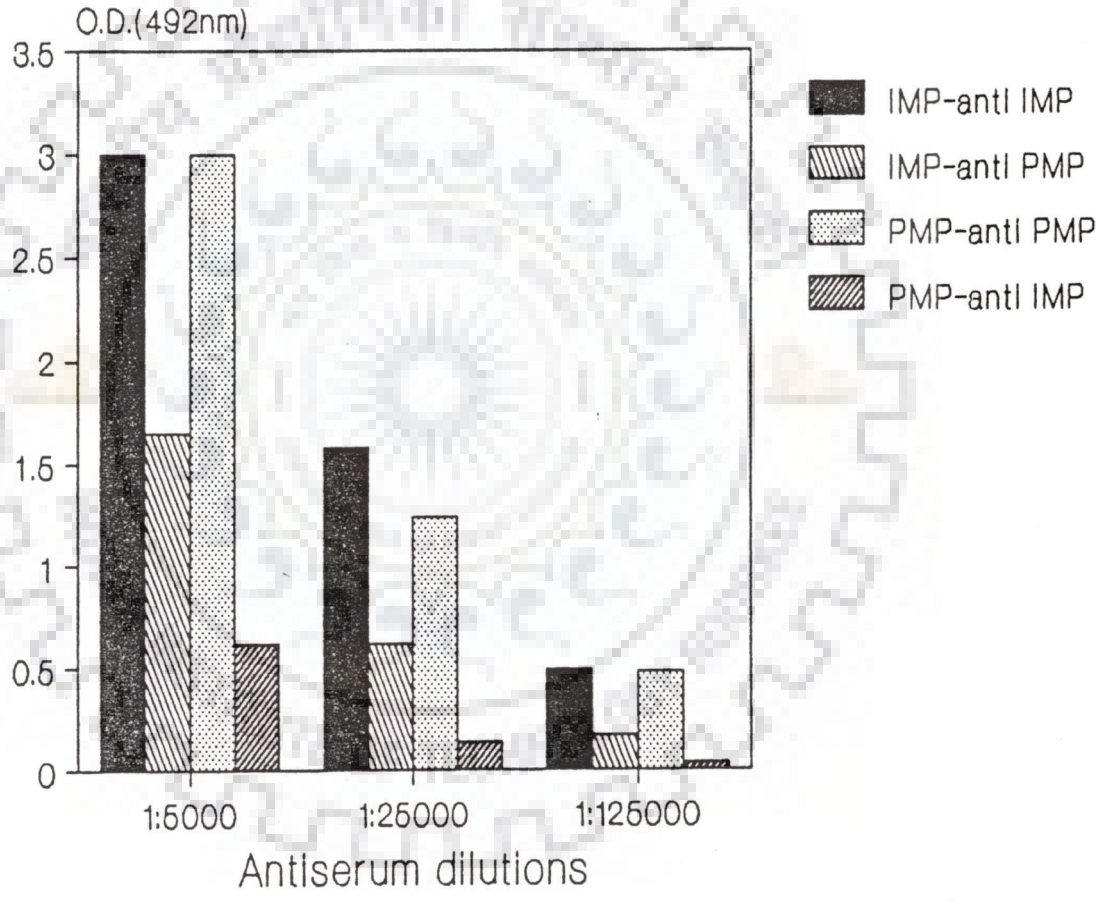

Fig 3.1(b) Serological specificity of integral and peripheral membrane proteins of BCG as determined by ELISA.

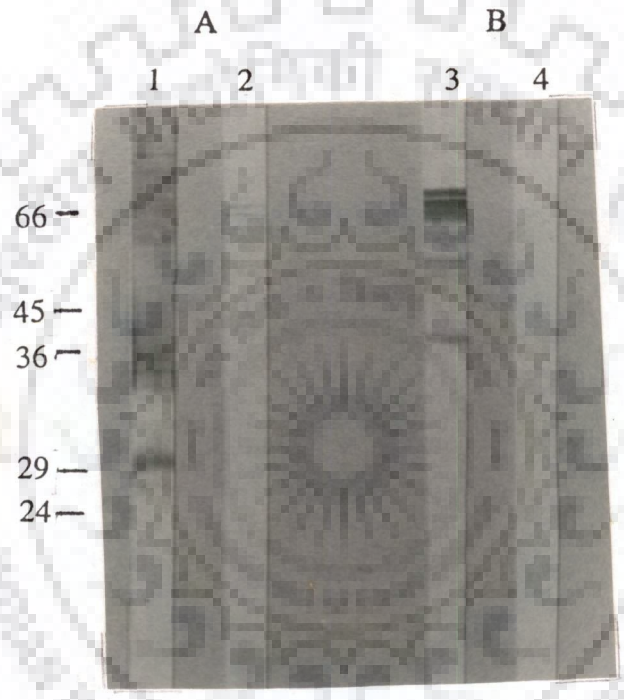

Fig. 3.2 Immunoblot of integral (A) and peripheral (B) membrane proteins using rabbit antisera (1:1000). Lanes 1 and 4 were probed with anti-IMP antibodies and lanes 2 and 3 with anti-PMP antibodies.

Fig. 3.3 Immunoblot showing antigenic overlap between IMPs of the slow grower, BCG (A) and the fast grower, *M. fortuitum* (B) using antisera raised in rabbits (1:1000). Lanes 1 and 4 were probed with anti-BCG-IMP antiserum and lanes 2 and 3 with anti-*M. fortuitum*-IMP antiserum.

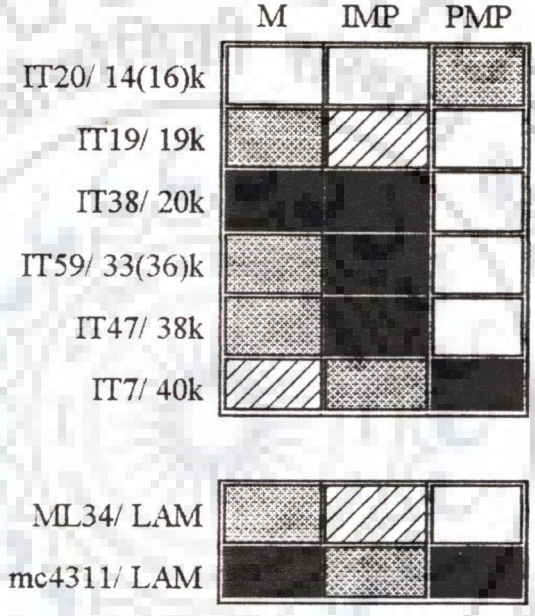

Fig. 3.4 Antigenic definition of proteins of BCG membrane (M) and its subfractions IMP and PMP using a panel of anti- mycobacterial monoclonal antibodies (Table 3.2). O.D. as determined by ELISA : ▱ <0.1; ▱ 0.1 - 0.2; ▨ 0.2 - 0.4; ■ >0.4,

0.2) only in whole membrane. Some important antigens which were absent in membrane or its subfractions were: heat-shock proteins (HSP) of 10, 18 and 70 kDa, superoxide dismutase (23/28 kDa) and antigen 85 (~30 kDa).

These results also exemplified the way moAbs could be used not only for identification of antigens but also for their definition. The IT47 epitope of 38 kDa protein was present in BCG but its IT21 and IT65 epitopes were absent. Similarly, the TB78 epitope of (M. tuberculosis) HSP 65 was present but the mc4343 and mc5205 epitopes (both of M. leprae hsp 65) were not.

3.3.2. Antegenic characterisation of LAM

In the previous chapter, presence of LAM in subcellular fractions of BCG was ascertained using a modified silver staining protocol (which preferentially stains glycoconjugates) for gels after SDS-PAGE. Further, another attempt to locate and antigenically characterize LAM was made by employing ELISA using three anti-LAM moAbs (MLO2, ML34 and mc4311) produced against three cross-reactive epitopes of M. leprae LAM. The moAb MLO2 did not react with BCG LAM. However, an interesting picture emerged with moAbs ML34 and mc4311 (confirmed by using different batches of BCG subcellular fractions). The epitope corresponding to ML34 was seen exclusively in the detergent soluble LAM (partitioning with IMP, OD= 0.32) whereas the mc4311 epitope was noticed mainly in the water soluble LAM (partitioning with PMP, OD= 0.65) Fig.3.4.

3.3.3. Human T cell proliferative responses to membrane antigens

In order to facilitate a comparative evaluation of T cell proliferative responses, the study subjects were selected mostly from those who are generally regarded as responders for mycobacterial antigens (Kaufmann, 1993; Ridley and Jopling, 1966). Table 3.2 shows proliferative responses expressed as Δ cpm (cpm with antigen or mitogen - cpm with medium alone). PHA, a mitogen, causing non-specific stimulation of T cells, was used as positive control in all cultures. The optimum concentration for

PHA (1 µg/ml culture) was already worked out in the laboratory and used routinely. However, the optimal concentrations for BCG antigens were determined separately in pilot experiments. They worked out to be 25 µg/ml for cytosol and 12.5 µg/ml for membrane, IMP and PMP.

The tested 20 subjects could be grouped as 'high' (n=12) or 'low' (n=8) responders depending on their response to membrane (M), using a Δ cpm cut off value as 5000 (Table 3.2). In all the high responders, the response to IMP (mean value 34341 ± 4558) was much greater than that with the whole membrane (mean value 208105 ± 2797), whereas in low responders, it (mean value 3872 ± 949) was almost equal to the whole membrane (3063 ± 503). Nonetheless, in both categories the proliferative T cell response to IMP was remarkably higher than that of PMP (the latter being almost nil in two cases). The immunodominance of IMP is also clearly evident from Fig. 3.5. Even though there was a wide variation in the individual responses, a grouped statistical analysis clearly brought out the distinction between responses with M, IMP and PMP. Δ cpm with IMP (group mean 23230 ± 19771) was significantly higher ($P < 0.0001$) than that with M (14272 ± 11630) or PMP (6984 ± 8227).

3.4. DISCUSSION

A clear segregation of integral and peripheral membrane proteins of BCG is evident from the results of ELISA using polyclonal as well as monoclonal antibodies which reestablished the robustness and precision of Triton X-114 based phase separation protocol (Bordier, 1981). Some cross- reactivity between IMP and PMP as seen at higher antiserum concentrations (Fig. 3.1b) could be attributed to LAM which was found to be present in both the membrane subfractions (Fig. 3.4) thereby generating anti-LAM antibodies in rabbit immunised with either IMP or PMP. Not all prominently stained proteins of IMP and PMP pool (as seen in SDS-PAGE, Fig. 2.4) were serologically active. It is quite likely that many of them are inducers of T cell response only. Furthermore, B-cell (or T-cell) epitopes are known to be manifested selectively in different animal species or human subjects. 248386,

Table 3.2 Human T lymphocyte proliferative responses

Subjects	Control		BCG		
	− (medium)	+ (PHA)*	M	IMP	PMP
a. High responders					
P1	1264	65660!	33164	59739	16500
P2	1467	43658	30261	49127	6891
P3	274	48265	19650	34024	18927
P4	226	26316	25846	16209	5745
P5	713	ND	16376	23584	7704
P6	472	60009	12685	16209	5745
P7	2634	4990	7258	12595	7329
P8	1559	23133	22633	35889	7681
P9	177	53353	27964	37982	29969
P10	501	43255	34531	48905	12594
HC1	711	139730	10529	52278	517
HC2	266	34255	8829	25556	1341
			208105	34341	10272
			±2797	±4558	±2302
b. Low responders					
P11	709	39290	3391	2219	2144
P12	462	23805	1909	4375	180
P13	334	19175	925	991	629
P14	357	90600	4764	8878	472
P15	92	93156	2356	2796	0
P16	342	27120	ND	2020	0
P17	446	53683	3508	6666	1007
HC3	134	34255	4588	3030	2531
			3063	3872	1161
			±503	±949	±342

* Cultures were set at optimum antigen/mitogen doses = 2μg/ml for phytohaemagglutinin (PHA) and 12.5 μg/ml for membrane (M), integral or peripheral membrane proteins (IMP and PMP respectively).

! Mean Δ cpm (cpm Exp. — cpm Medium) of triplicate cultures. S.E.M. in each case was < 15% of mean. Group mean for all 20 subjects : M=14272±11630; IMP=23230±19771; PMP=6984±8227. 'P' (IMP vs M or PMP) <0.0001.

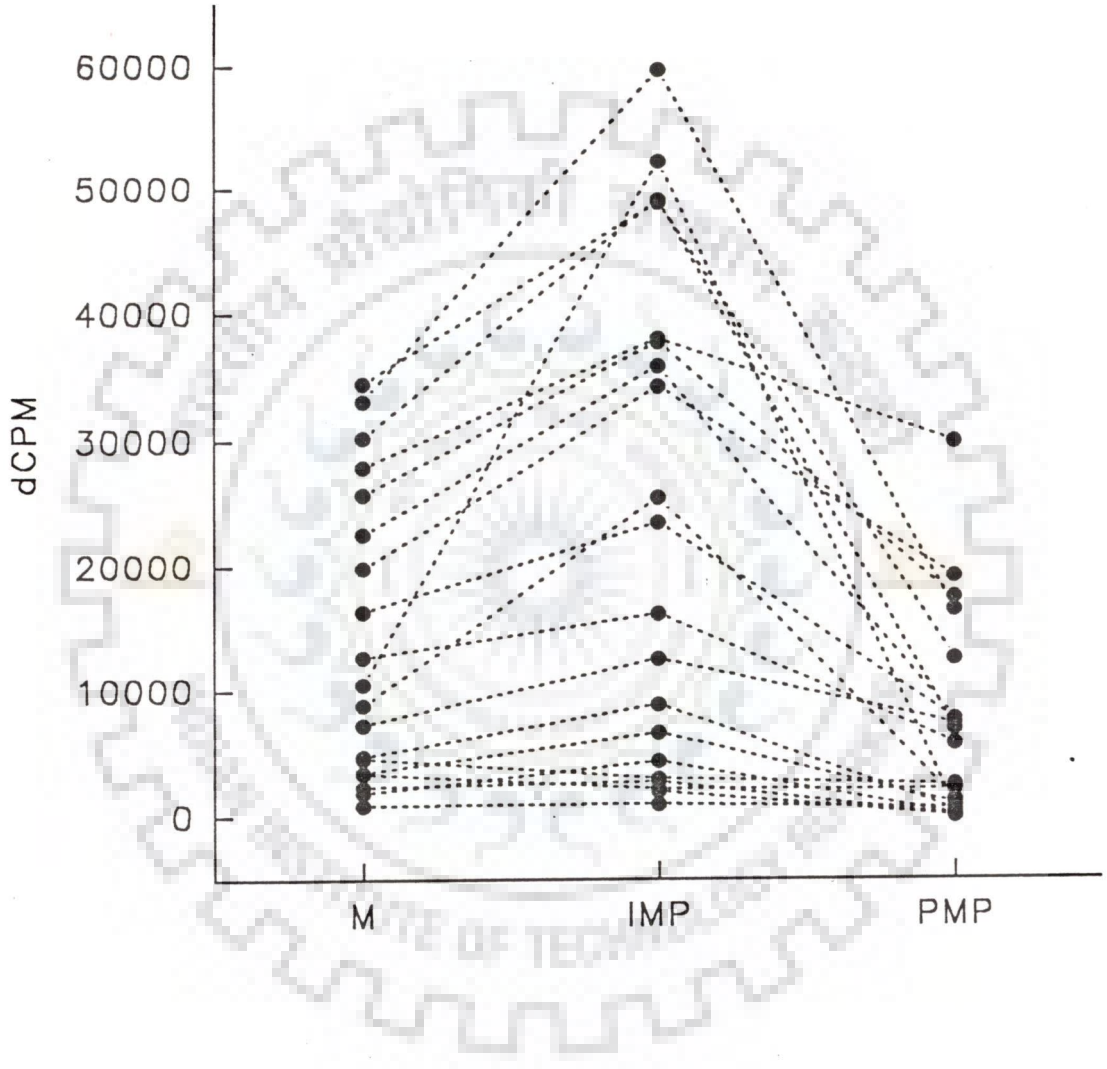

Fig. 3.5 Proliferative T cell responses with BCG - membrane (M) and its subfractions (IMP and PMP) in 20 subjects (17 tuberculoid leprosy patients and 3 healthy volunteers). Individual responses (Δ cpm denoted in table 3.3) have been traced with dotted lines.

BCG-IMP when cross-reacted with anti-IMP antiserum of the fast grower M. fortuitum (or vice versa) revealed very little overlap between the two antigen classes. Even the pattern of seroreactive bands was quite different. Using the lesser sensitive 'diffusion in gel' technique, Stanford and Grange (1974) have classified seroreactive mycobacterial antigens into four categories: (a) shared by all species, (b) shared amongst slow or (c) fast growers and (d) species specific antigens. Thus, the distinction observed by us using the more sensitive and definitive immunoblotting technique could largely be due to (b),(c) or (d) categories of antigens. However, 'shared by all' (category-a) antigens were least conspicuous. Clearly, a detailed study, employing contemporary techniques, is warranted for comprehensive classification of mycobacterial antigens.

Earlier work on membrane proteins of M. leprae (Hunter et al., 1990) and M. tuberculosis (Lee et al., 1992) did not include an extensive screening for the presence of previously known mycobacterial antigens, nor were the membrane proteins evaluated for their human T cell activating potentials. The studies described isolation and characterisation of two PMP from M.leprae and another such protein from M. tuberculosis. The 35 kDa M. leprae protein which reacted with a previously described moAbs (Hunter et al., 1990; Ivanyi et al., 1985) was found responsible for generation of high titres of specific antibodies in leprosy patients, thus having implications for diagnosis and patient care (Ivanyi et al., 1985; Chaturvedi et al., 1991). However, an attempt to vaccinate mice with 35 kDa protein against foot pad challenge with M. leprae did not succeed (Gelber et al., 1994), despite earlier encouraging results (Gelber et al., 1990). The 22 kDa M. leprae protein was further characterised as a bacterioferritin possibly playing the role of a virulence factor (Pessolani et al., 1994). This protein also did not protect mice against foot pad challenge (Gelber et al., 1994). The 16 kDa M.tuberculosis protein, on the other hand, was characterised as a novel antigen capable of eliciting antibody responses in most pulmonary tuberculosis patients (Lee et al., 1992).

An interesting profile of membrane protein of BCG emerged from the pattern

of their reactivity with a pannel of IMMYC moAbs against cross-reactive epitopes of mycobacterial antigens. Out of the 17 previously described antigens, BCG membrane and its subfractions showed positivity for 7, including LAM. Three well characterised mycobacterial protein antigens of 19 kDa, 33/36 kDa ('proline rich') and 38 kDa (Pst S homolog) were amongst the IMPs of BCG and another such antigen, 14/16 kDa α-crystallin homolog (a low mol wt heat-shock protein, hsp) constituted a part of the PMP. A 40 kDa antigen partitioned into both IMP and PMP pools with a preference for the latter. While the 14/16 kDa water soluble antigen was earlier reported to be a PMP (Young and Garbe 1991; Lee et al., 1992), the other antigens were not identified as membrane proteins.

Earlier, only Young and Garbe (1991) had attempted to characterise the 'detergent soluble' proteins from M. tuberculosis. They described the 19 kDa and 38 kDa proteins as lipoylated, detergent soluble molecules present in the sediment of the lysate as well as culture filtrate of M.tuberculosis. Both proteins, particularly the 38 kDa, have been found useful for sensitive and specific serodiagnosis of tuberculosis (Harboe and Wiker, 1992). The fact that patients make high titres of antibodies to these molecules is corroborated by the observation that both induce a Th2 type of response (Surcel et al., 1994). Even though human proliferative T cell response to native 38 kDa protein is not high, large scale clinical trials were undertaken with the purified antigen 5 (38 kDa) as a skin test reagent for diagnosis of tuberculosis. Initial results generated high hopes which remained unsubstantiated (Daniel et al., 1982). Human T cell response to the recombinant 19 kDa protein is also low (Surcel et al., 1994) and an attempt to vaccinate mice with recombinant M. vaccae expressing this antigen did not succeed (Abou-Zeid et al., 1997). The 33/36 kDa 'Proline rich antigen' was originally described in M. leprae with the help of species specific and cross-reactive moAbs (Thole et al., 1990) It has shown an impressive serodiagnostic potential with high specificity and sensitivity for leprosy. Isolation and characterisation of a 'Proline rich antigen' from culture filtrate of BCG has also been reported, though its homology with that of M. leprae was not

explored (Romain et al., 1993). The antigen bears T cell epitopes as evidenced by existence of human T cell clones (Ottenhoff et al., 1986), but a 36 kDa antigen based DNA vaccine provided relatively poor protection in mice (Tascon et al., 1996).

An antigenic description of the remaining two moAb reactive BCG-IMPs, 20 kDa and 40 kDa, is not available in literature. Nonetheless, considering the similarities in size and partitioning behaviour, the possiblity of 20 kDa and 19 kDa proteins being one and the same can not be excluded. The 40 kDa antigen could be the previously described alanine dehydrogenase (Andersen et al., 1992). The 14/16 kDa α-crystallin homolog present in the BCG-PMP pool is also known to bear both B and T cell epitopes but corresponding responses in tuberculosis patients were not remarkable (Mathews et al., 1985).

Localisation of immunosuppressive LAM and its antigenic characterisation formed an important part of this study. The relevance of LAM is due to its various immunosuppressive and potentially pathogenic properties described prominently in literature (Berman et al., 1996; Moreno et al., 1988; Chatterjee et al., 1992; Sibley et al., 1990). It is basically a component of mycobacterial cell wall, but also constitutes a significant part of membrane to which it is anchored via its lipidic tail, PIM (Fig. 1.2). Contrary to the observation of Mehrotra et al. (1995) with M. fortuitum where membrane LAM partitioned exclusively into the aqueous phase (together with PMP), the LAM moieties of BCG membrane going into aqueous and detergent phases appeared to be antigenically different. Their selective reactivity with moAbs against two different epitopes of M. leprae LAM could be a reflection of the well documented structural heterogeneity of LAM as seen in from different mycobacterial species or even within the same species (Leopold and Fischer, 1993). Data on antigenic characterisation and comparative estimates of LAM in different species of mycobacteria are not available.

The detergent phase partitioning of membrane LAM in M.leprae and M.tuberculosis has been reported by earlier workers (Hunter et al., 1990 and Lee et

al., 1992). But these observations were based only on differential staining of gels which may not be the conclusive evidence for its precise location. In case of BCG also, we could not detect LAM in the aqueous phase (PMP fraction) by staining method (Fig.2.5 in chapter 2), though its presence in PMP was very clearly evidenced by the more sensitive ELISA technique (Fig. 3.4).

Despite the presence of LAM, IMP of BCG emerged as a strong inducer of T cell proliferative responses in subjects primed with mycobacterial antigens (Table 3.2). These results were consistent with the observations of Mehrotra et al. on M.fortuitum (1995). Though, we could not quantitate LAM in the BCG membrane or its subfractions, its concentration was apparently lesser than that required (30 µg/ml) for a suppression of T cell proliferation. All the tested subjects showed impressively higher response to the IMP than PMP (Fig.3.5). A potent T cell stimulation in all the responder subjects from a heterogeneous MHC background makes IMPs promising candidates for designing 'universally' effective control strategies such as diagnostics and vaccines.

Promiscous binding of certain peptides to different MHC specificities is a well known phenomenon which essentially requires amino acids with a hydrophobic propensity at the conserved 'anchor' positions (Hammer et al, 1993). IMP could be an ideal source for such peptides. It is needful to emphasize here that despite the serological disparity between the slow grower BCG and the fast grower M.fortuitum (Fig. 3.2), IMPs of both these mycobacteria were equally potent in stimulating T cells from responder subjects belonging to a varied MHC background. Thus, the clue to such high responsiveness could lie in shared proteins structures rather than shared antigenicity. Lipid modification a feature of IMPs (Chamberlain et al., 1989), has been associated with high antigenicity particularly for T cells (Deres et al., 1989, Rees et al., 1993). Young and Garbe (1991) have focused attention on 4 immunodominant lipoproteins of M.tuberculosis at least two of which (19 and 38 kDa) happen to be the constituents of IMP. It needs to be seen how many more (or all) IMPs are lipidated.

The contribution of previously described immunodominant antigens (19, 33/36and 38 kDa) towards immunodominance of BCG-IMP needs to be considered. Two of them (19 kDa and 38 kDa) were not detectable in M.fortuitum IMP which was found to induce strong T cell activity (Mehrotra et al., 1995) comparable to what is observed with BCG. However, the fact that the three IMPs (19 kDa, 33/36 kDa, 38 kDa) are also 'secretory' (Young and Garbe, 1991; Romain et al., 1993) raises an interesting possibility that the observed immunodominance of secretory antigens (Boesen et al., 1995) could atleast partly be due to the proteins overlapping with IMPs. The extent of such overlap needs to be explored. However, it appears to be only partial since one of the most immunogenic secretory proteins, the 30/31 kDa antigen 85, could not be detected in the BCG membrane; though its presence in the cell wall (Harth et al., 1996) was evident from ELISA using the moAb HYT 27.

CHAPTER IV

IDENTIFICATION OF INTEGRAL MEMBRANE PROTEINS BEARING IMMUNODOMINANCE FOR HUMAN T CELLS

4.1 INTRODUCTION

T cell mediated immune responses determine the natural course of infections due to intracellular parasites such as mycobacteria (Kaufmann, 1993). Thus T cell activating proteins of these microbes continue to generate active interest particularly in view of their possible role in the design and development of newer and more effective diagnostic tests and vaccines. An impressive array of mycobacterial proteins bearing antigenicity for T cells has been identified and characterised (Young et al., 1992 and Thole et al., 1995). Nonetheless, since these proteins have mostly been picked up from crude extracts by biochemical means or from recombinant DNA libraries with the help of antibodies, it is quite likely that many predominantly T cell activating molecules have been missed. This realisation has led to a series of attempts towards development of appropriate technology which would ensure a comprehensive search for such molecules (Abou-Zied et al., 1987; Gulle et al., 1990; Andersen and Heron, 1993; Mountford and Wilson, 1993).

An important advancement in this direction was the technique of "celloblot" in which the constituent proteins of a crude mixture were resolved by SDS-PAGE, electroblotted on nitrocellulose membrane and slices of the membrane were used as such or after pulverisation for the evaluation of T cell activity (Young and Lamb, 1986; Abou-Zeid et al., 1987). However, this technique could not get widespread recognition due to certain inherent problems such as low yields imposing restrictions on the number of experiments, batch to batch variations, and a possible masking of epitopes due to attachment onto nitrocellulose. Simultaneous electroelution following two dimensional native PAGE represented an improvement in the sense that it permitted greater resolution and fraction could be recovered in a solution form (Gulle et al., 1990). However, the yields were too low to undertake meaningful investigations. The technique of "high performance electrophoretic chromatography" (HPEC) and "simultaneous electroelution" which are based on one-dimensional SDS-PAGE (Anderson and Heron, 1993; Mountford and Wilson, 1993) ensure greater recoveries,

albeit within limits. Efficient removal of SDS and monitoring of its concentrations so that they remain within biologically safe limits is also an important consideration in the development of a purification strategy based on SDS-PAGE. Though earlier reports have incorporated protocols for SDS removal, assessment of success of such protocols was restricted to trial and error without any quantitative estimates.

In our laboratory we have recently developed a continuous elution SDS-PAGE protocol (Mehrotra et al., 1997) which allows large scale fractionation/purification of detergent soluble protein mixtures and their recoveries in immunologically active form. Using this technique the BCG-IMPs could be comprehensively screened by human T cell proliferation assays enabling the precise identification of 3 low molecular weight immunodominant subunits.

This chapter discusses in detail the continuous elution SDS-PAGE protocol and its advantage over existing techniques.

4.2 MATERIALS AND METHODS

4.2.1 Fractionation of integral membrane proteins of BCG by continuous elution SDS-PAGE

Requirements

All reagents for SDS-PAGE and silver staining were prepared as described previously in chapter II (sections 2.2.6 and 2.2.7).

Elution buffer : 0.025 M Tris-HCl containing 0.1% SDS, pH 8.3.

Integral membrane proteins (IMPs) : Prepared as described previously (chapter II, section 2.2.4). 5 mg IMP was lyophilised in a freeze drier.

Procedure

Fractionation of IMPs was done by continuous elution SDS-PAGE using Laemmli (1970) buffer system in a 'Prep Cell' (Model 491, Bio-Rad, USA, Fig. 4.1). Prior to this, a resolving gel concentration suitable for the whole range of proteins was

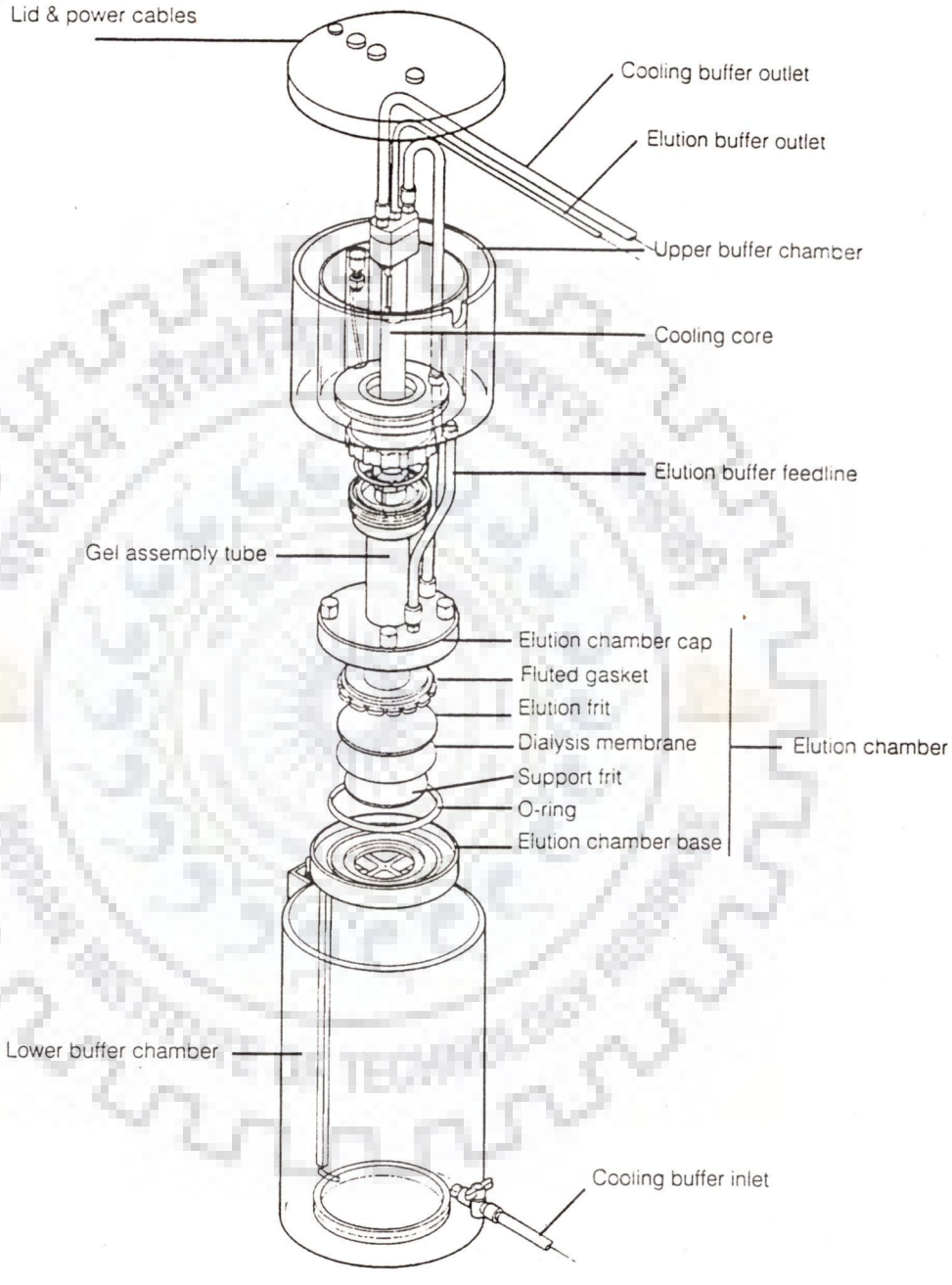

Fig. 4.1 Model 491 Prep Cell Components

determined by running a series of SDS-PAGE on mini gel slabs (Broviga, Chennai, India). Accordingly, 10% resolving gel (height, 8 cms) was cast in a 27 mm diameter tube. The gel surface was overlayed with butanol saturated with water and left to polymerise for 3-4 hours at room temperature. Water was circulated through the cooling core (Fig. 4.1) to maintain the temperature. Butanol was then removed with the help of a syringe and after washing the surface 2-3 times with water it was covered with 2-3 ml of resolving gel buffer and left overnight for polymerisation at 20-22°C. Next morning the buffer was removed from the gel surface and a 4% stacking gel (height 1.5 cm) was poured and left to polymerise for 1-2 hours. After assembling the apparatus according to supplier's instructions, 5 mg lyophilised IMP was solubilised in sample loading buffer (containing 2 mercaptoethanol) by keeping in boiling water for 5 min and loaded on the gel column. Electrophoresis was conducted at 12 Watts for 9 hours and, during this period, elution buffer was pumped at a flow rate of 1 ml/min. 111 x 3 ml fractions were collected starting immediately after the elution of dye front. It was not possible to monitor the elution profile by UV absorbance at 280 nm, as the chart recorder could not discriminate between individual peaks probably due to their close proximity. Consequently, each fraction was analysed on mini gel slabs and silver stained to visualize the protein bands . Tubes containing identical bands were pooled to provide 15 discrete fractions.

4.2.2 SDS estimation in eluted IMP fractions

The colorimetric SDS estimation method of Arand et al. (1992) was followed.

Requirements

Methylene blue reagent : 5g anhydrous sodium sulfate was dissolved in 90 ml of TDW. 1ml of concentrated H_2S0_4 was mixed gently and 25 mg methylene blue added to it. Final volume was raised to 100 ml with TDW. The reagent was stored at room temperature.

SDS stock solution : 0.1 mg/ml was prepared for plotting the standard curve.

Anhydrous sodium sulfate powder

Chloroform

Procedure

For estimation of SDS in eluted protein fractions, a standard curve was plotted (Fig.4.2) with varying concentrations (0.3 µg to 3 µg) of SDS taken in duplicate in eppendorff tubes. The final volume was made upto 100 µl with TDW in each tube. 300 µl of methylene blue reagent and 1.2 ml of chloroform was added to it and mixed properly. The tubes were centrifuged at 10,000 g for 2 min and upper coloured layer was removed. 50-100 mg of anhydrous sodium sulfate was added and mixed by inverting the tubes to remove traces of water. It was centrifuged for 3-4 min as above, and OD of supernatant was read at 651 nm, using chloroform as reference. In the case of eluted fractions, an appropriate volume was taken and processed as above. SDS concentration in the unknown samples was estimated from the curve.

4.2.3 SDS removal from the eluted IMP-fractions

A method of Wessel and Flugge (1984) was adopted

Requirements

Methanol

Chloroform

Acetone

PBS : as described in chapter II, section 2.2.3

Procedure

All the 15 fractions were concentrated on a speed vac (Savant, U.S.A.) and aliquoted (0.1 ml) in 2 ml eppendorff tubes. 400 µl of methanol was added and mixed gently, followed by addition of 100 µl of chloroform and 300 µl of TDW. After vortexing the tubes were spinned at 10,000 rpm for 5 min (at 20-25°C) to separate the two phases. The upper aqueous layer was carefully removed. To the remaining

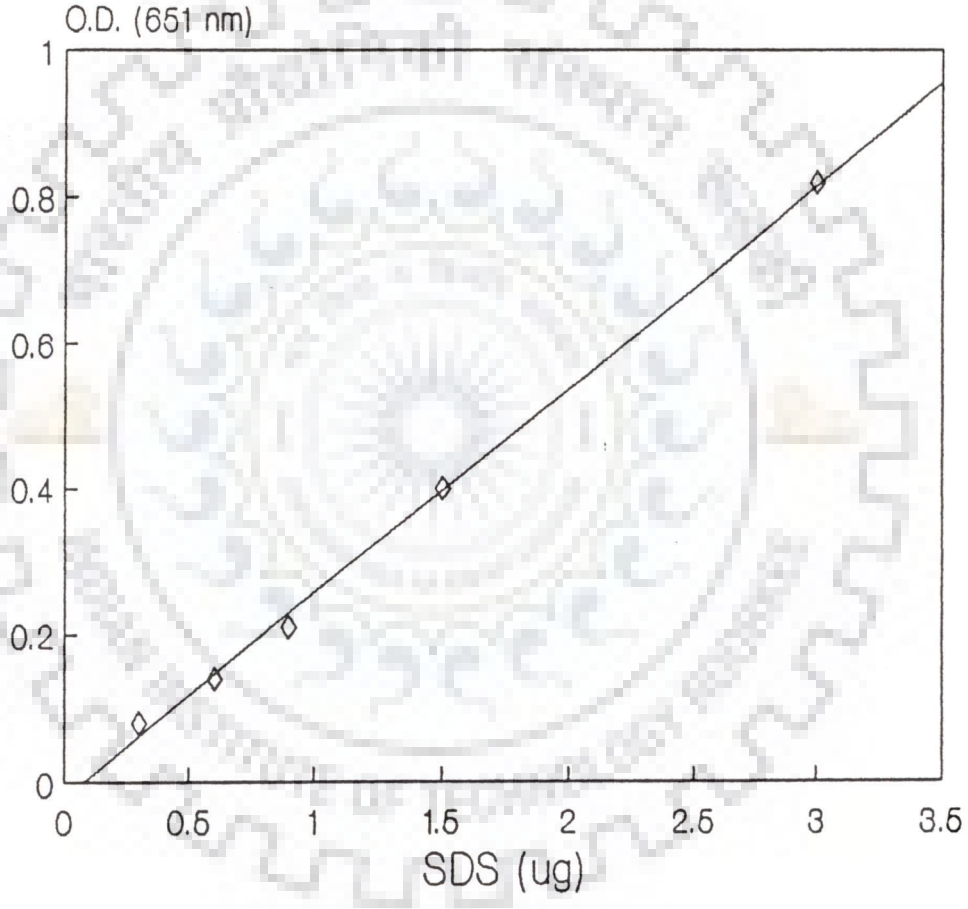

Fig. 4.2 Standard curve for colorimetric estimation of SDS.

solution methanol, chloroform and water were added again as above and the procedure repeated to ensure proper removal of SDS. The protein from the lower layer was precipitated with methanol (300 µl). The precipitates were sedimented (15,000 g for 15 min), washed with chilled acetone, air dried and reconstituted (0.5 ml/fraction) in PBS. Samples thus prepared were autoclaved and stored at 4°C for use in tissue culture experiments. A part (~100 µl) of each fraction was stored separately for doing ELISA to detect the presence of LAM as described below.

4.2.4 Identification of LAM containing fractions

The presence of LAM in BCG-IMP fractions was monitored by ELISA using moAb ML34. The reagents used and procedure followed were as described in chapter III, section 3.2.1.

4.2.5 Human T cell proliferation assay

Study subjects : Five subjects were selected from patients evaluated for their T cell responses to whole IMPs, as mentioned in chapter III, section 3.2.3.

Requirements

RPMI, PNS, Ficoll-Isopaque, scintillation fluid, ^{3}H-thymidine and heparin were prepared as mentioned in chapter III, section 3.2.3.

Antigens : autoclaved IMP fractions after SDS removal (as mentioned in section 4.2.3).

Procedure

As described in chapter III, Section 3.2.3

4.3 RESULTS :

4.3.1 Fractionation profile of IMPs

The BCG IMPs consisted of a range of proteins, mostly with molecular masses below 70 kDa. All proteins upto ~56 kDa could be resolved and recovered into 15 distinct fractions, under the used experimental conditions (Fig.4.3). Different elution

buffer, flow rates and fraction sizes were tried in pilot experiments before adopting 1 ml/min and 3 ml/fraction, which produced recoveries with least overlaps. Most of the fractions contained more than one protein although some of them, particularly in the higher molecular weight range, appeared to contain single bands. Average molecular weight of each fraction (Fig. 4.3) was extrapolated from a curve drawn by using standard molecular weight markers (as previously described in chapter II, section 2.2.7).

4.3.2 SDS removal and its inhibitory concentration for cell proliferation

SDS content of fractions before and after its removal was estimated to be in the range of 1-1.5 mg/ml and 5-10 µg/ml, respectively, indicating at least a 100 fold reduction. Thus, the amount of 'residual' SDS going into cell cultures alongwith the antigen (described below, under 'T cell proliferative response') works out to be ≤1 µg/ml.

In order to assess the effect of SDS on cell proliferation, its graded amounts (final concentration/ml culture = 0, 5, 25 and 125 µg) were mixed and incubated (37°C, 1 h) with a predetermined dose (25 µg/ml culture) of mycobacterial antigen (BCG cytosol, prepared as described in chapter II, section 2.2.2; made sterile by passing through 0.2 µm syringe filter). Results on 5 subjects are shown in Fig. 4.4.

In all cases, SDS upto 5 µg/ml did not produce any significant change in the proliferative response ($P > 0.05$, by 't' test) where as 25 µg/ml SDS significantly inhibited the response in 4 subjects ($P < 0.05$). The dose of 125 µg/ml was drastically inhibitory in each case. These results indicate that the residual SDS present alongwith fractionated antigens (equivalent to ≤ 1 µg/ml culture) was well below its inhibitory concentration.

4.3.3 T cell proliferative responses to fractionated IMPs for identification of immunodominant subunit(s)

For human T cell proliferation assays, all fractions, after SDS removal, were reconstituted in identical volumes (0.5 ml) in order to maintain their relative concentrations as present in the unfractionated IMP pool so as to facilitate an

Fig. 4.3 Fractionation ladder of BCG-IMP after continuous elution SDS-PAGE. Each fraction (A-O) was analysed on SDS-PAGE mini gel (12%) slabs and visualised by coomassie blue staining.

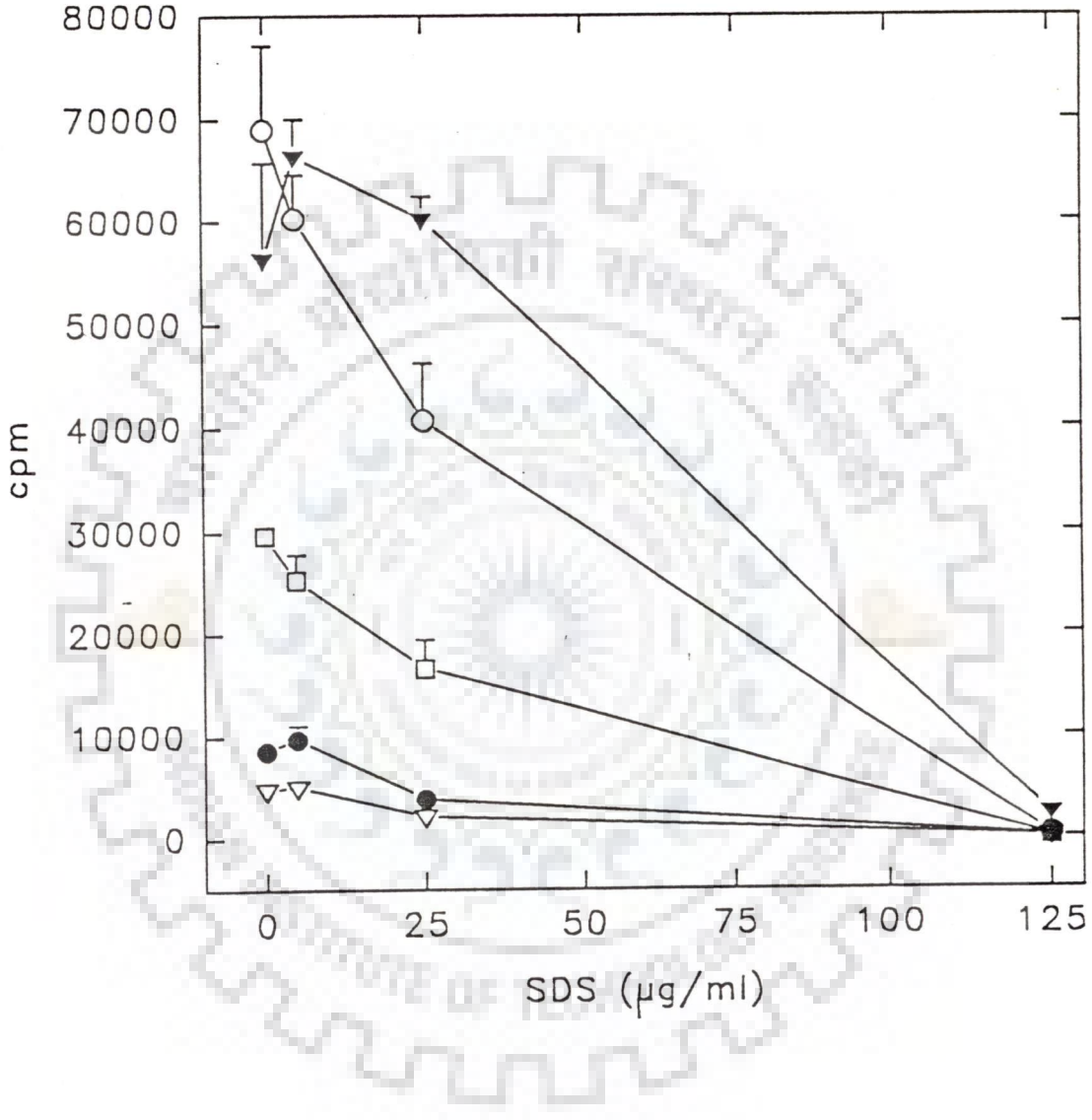

Fig. 4.4 Tolerable dose of SDS in human T cell proliferation assay. The response (mean cpm ± SE) of 5 subjects with BCG cytosol (25 µg/ml culture) preincubated with or without SDS (concentration/ml culture : 0, 5, 25 and 125 µg) is dipicted by individual curves. In all cases, except one (–▼–), the dose 25 µg / ml was significantly ($P < 0.05$) inhibitory.

'unbiased' comparasion of their relative potencies. Different doses (5, 10 and 20 µl/well) of each fraction were tried to arrive at a conclusion that 10 µl/well (50 µl/ml) was suitable in the case of BCG.

The results on 5 subjects (from those included in table 3.2) are depicted in Fig. 4.5. The proteins of a low molecular weight range (< 35 kDa) were mainly found to be involved in T cell stimulation. Most active subunits comprised 3 immunodominant zones with average molecular weights of 16-17, 22 and 30-33 kDa, of which the 22 kDa protein(s) induced response in all subjects. Remarkably, in 4 out of 5 cases, this response (SI) was > 50% of that observed with whole IMP. The antigens with a relatively higher molecular weight (fractions J to O) were of little or no consequence in this respect. The elution profile of LAM was also monitored in these fractions by ELISA (Fig. 4.6) using the moAb ML34. Fractions J-L (40-45 kDa range) showed positivity (average OD = 0.18).

4.4 DISCUSSION

The continuous elution SDS-PAGE protocol developed in our laboratory (Mehrotra et al., 1997) could be successfully used for the fractionation of BCG-IMPs. The 'Prep Cell' design for continuous elution electrophoresis offers following advantages: **a)** It can accomodate a sufficiently large amount of sample so as to meet the requirements for population based studies, and **b)** it offers a 'flexibile' resolution (from gross fractions to pure molecules, according to the need) while ensuring recoveries without losses. Though we have presently used 5 mg protein, the system is capable of fractionating upto 500 mg sample load. The 10% resolving gel used by us did not seggregate low molecular weight (< 30 kDa) bands with the precision seen in the case of high molecular weight bands.

Nonetheless, in accordance with the principle of SDS-PAGE, it should be possible to resolve and recover the lower bands with homogeneous purity by using a suitably higher gel concentration. Likewise it is also possible to recover bands above 56 kDa, using a lower gel concentration. These possibilities also exist with another continuous

elution electrophoresis system, HPEC (Mountford and Wilson, 1993), except that it has a significant limitation in terms of the amount of sample which could be fractionated in a single run. The technique of 'simultaneous electroelution' from gel slabs following SDS-PAGE (Andersen and Heron, 1993) has been used extensively for fractination and screening of T cell stimulating proteins from mycobacterial culture filtrates (Boesen et al, 1995). It permits a higher sample load, though much less than that attainable with the Prep Cell. Further, since the technique is designed to provide 'sharply defined protein fractions within narrow molecular mass ranges' (Andersen and Heron, 1993) as elution is performed on a template of prefabricated slots, it may fall short of yielding homogeneously pure proteins.

Quantitative estimation of SDS in fractions before or after the removal process was not attempted in earlier reports (Andersen and Heron, 1993; Mountford and Wilson, 1993). At our hands, the colorimetric method of Arand et al. (1992) worked with desirable sensitivity for monitoring SDS levels. The SDS removal protocol of Wessel and Flugge (1984) was found to be most convenient as it worked efficiently even though each fraction (along with its SDS contents) was concentrated several folds. Prior to this, we tried SDS removal by two other methods (data not shown) : (a) Dialysis, which led to precipitation of SDS at 4°C and microbial contamination if carried out at room temperature. In addition, the number and size of samples were too large to handle. (b) ethanol precipitation (Mountford and Wilson, 1993), which resulted in incomplete removal of SDS.

The use of continuous elution SDS-PAGE permitted identification of immunodominant BCG-IMP subunits which could be mapped to three 'low molecular weight' regions. The immunodominance of low molecular weight membrane proteins for human T cells as observed by us previously with M. fortuitum (Fig. 4.7a and b) and presently with BCG (Fig. 4.5) corroborates earlier reports showing prominent T-cell activation by low molecular weight mycobacterial antigens, although the studied antigens were cytosolic/secretory rather than membranous (Andersen and Heron,

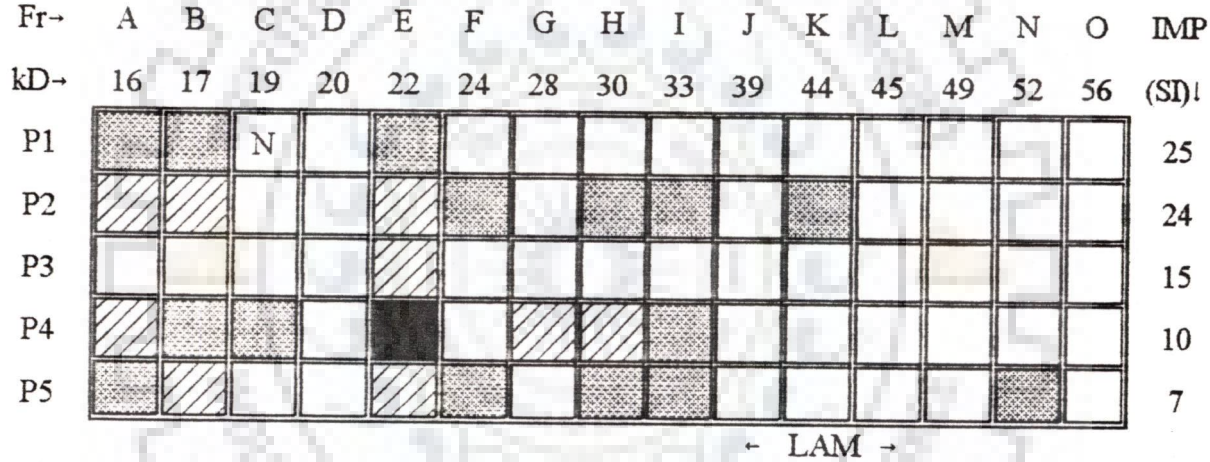

Fig. 4.5 Proliferative T cell responses to BCG-IMP fractions (A - O, av. mol. wt. 16-56 kDa) in tuberculoid leprosy patients (P1 - P5). Data represented as % stimulation index (100% being corresponding SI with whole IMP) [□], 0 - 25%; [▩], 25 - 50%; [▨], 50 - 75% ; [■], 75 - 100%. Fractions J - L (39-45 kDa) contained LAM (av. O.D., 0.18). N, not tested.

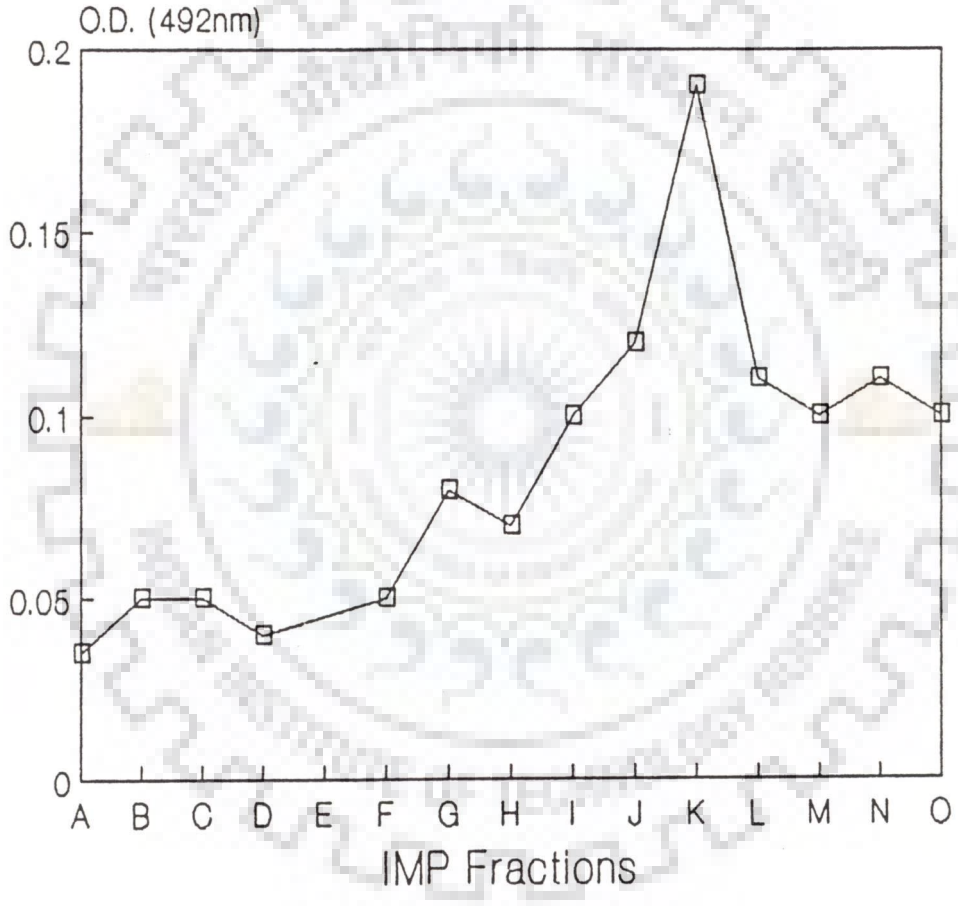

Fig. 4.6 Elution profile of lipoarabinomannan (LAM) in BCG-IMP fractions.

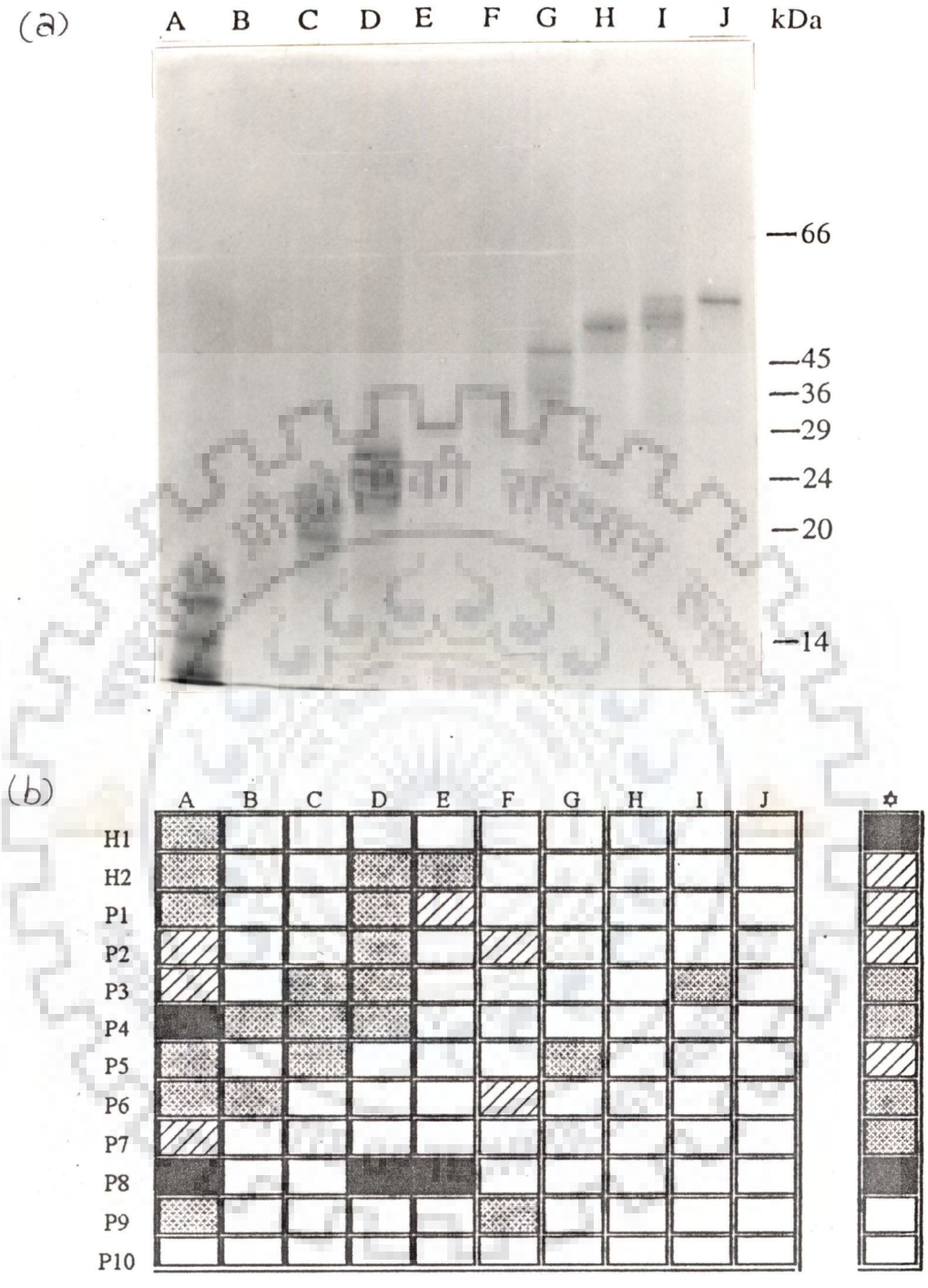

Fig. 4.7 (a) Protein profile (SDS - PAGE, 11% gel silver stain) of prep cell fractions of *M. fortuitum* IMP (lane A-J) and (b) their proliferative T cell responses in healthy contacts (H1 and H2) and tuberculoid patients (P1 - P10). Data represented as ranges of stimulation index (SI). In case of IMP(✡) SI < 3 ( ), 3 - 20 ( ), 20 - 30 ( ), and > 30( ); in case of fractions, SI < 3 ( ), 3 - 5 ( ), 5 - 7 ( ), > 7 ( ).

1993; Ottenhoff et al., 1989; Boesen et al., 1995; Sinha et al., 1987). Taken together, these observations suggest that the infection/exposure with mycobacteria results in a preferential recruitment and expansion of T cells bearing specificity for low molecular weight proteins. The BCG-IMP fractions (J-O) with relatively higher molecular weights (> 35 kDa) did not produce any significant response. Non-responsiveness with these fractions could perhaps be assigned to the presence of LAM which co-eluted with 40-45 kDa BCG-IMP fractions. However, a similar cut off of molecular weight was observed in our studies with M. fortuitum-IMP also (Fig. 4.7) which lacked LAM. This could be suggestive that some proteins are indeed dispensible in the quest for T cell activating subunits (Boesen et al., 1995). Extensive structural analysis of the low and high molecular weight IMPs and effect of structural variations (e.g. 'post translational' modification) on their immunogenicity could give a more definite justification for the observed difference in T cell responses.

The implications of the observation that some single IMP fractions could produce positive responses in almost all the study subjects in a manner akin to the whole IMP should be considered (Fig. 4.5). Apparently, some antigens can stimulate T cells from a cross-section of population by overcoming the restrictions imposed by an obvious heterogenieity in the MHC (Boesen et al., 1995; Germain, 1994). Of particular interest was one subunit of BCG-IMP (~22 kDa) which induced proliferative T cell response in all responders despite the obvious heterogenieity in their MHC background. Furthermore, the response to this single moiety was quite high, mostly 75% of that produced by whole unfractionated IMP.

The final proof for the "newness" and molecular identity of the immunodominant IMPs of BCG identified by us would emanate from database searches using partial sequences. Such analysis could also reveal their possible identity as enzymes, particularly those involved in cell wall synthesis (Belanger et al., 1996), for exploitation as new drug targets. This notwithstanding, the recognition of certain immunodominant 'low molecular weight' IMP subunits by T cells from a cross-section of human population makes them worthy of in-depth analysis from the view point of vaccination or diagnosis.

SUMMARY

The twin mycobacterial diseases - tuberculosis and leprosy continue to persist as major public health problems. Mycobacterium tuberculosis, the causative agent for tuberculosis, is responsible for an estimated 8 million new cases and 3 million deaths every year; whereas the present global prevalence of leprosy, caused by M.leprae,is around 6 million. A formidable worldwide resurgence of mycobacterial diseases, particularly tuberculosis in its deadly multidrug resistant (MDR) forms, is arousing even greater concern.

Primary prevention through active vaccination is considered as the ideal and most cost-effective strategy for control of communicable diseases. However in the absence of such option, diagnosis and effective chemotherapy are expected to restrict their prevalence. Failure of integral vaccines (i.e., based on whole bacterial cells), due partly to their immunosuppressive and inflammatory constituents has paved the way for rationally designed subunit vaccines.

A successful T cell mediated immune (CMI) response of the host is required to overcome the infections caused by intracellular parasites, including mycobacteria. The realisation that such responses (viz., Th1 type, leading to production of cytokine IFN-γ) are induced by proteins (or peptides) has led to an exquisite characterisation of mycobacterial protein antigens. However, despite the extensive search, quest for the desirable molecules remains unfulfilled. The proteins characterised till date belong to the mycobacterial cell wall, cytosol or culture filtrates; picked up either from recombinant DNA libraries with the help of monoclonal/polyclonal antibodies or by biochemical means. In this setting, the limitation of probes used/techniques adopted could have affected the identification of relevant T cell stimulating antigens.

There is considerable evidence suggesting immunodominance as well as vaccine potential of detergent soluble 'integral membrane proteins' (IMP) of various pathogens. A recent study from our laboratory has also found it true for mycobacteria. The immunodominance of IMP has been attributed to their inherent hydrophobicity and lipid modification.

This study is aimed at antigenic definition of plasma membrane proteins of BCG vaccine and identification of immunodominant T cell activating subunits. Selection of BCG was based on the premise that it has afforded considerable (though not upto satisfaction) protection against tuberculosis and more so against leprosy. Attempts to substitute this vaccine with a better alternative have not met with success. Apart from being clinically safe, BCG is also bestowed with adjuvant properties. In view of these advantages, it has now been entrusted a new role as 'vehicle' for immunoprotective molecules of various pathogens. Against this backdrop, an understanding of the immunodominant antigens of BCG is likely to provide clues to the development of more effective immunoprophylactic/immunotherapeutic agents based on cross-reactive mycobacterial antigens which have long been known to confer protection to the host.

Culture grown BCG (Indian vaccine strain) was subjected to sonication and differential centrifugation (according to established protocols) to isolate the plasma membrane which was further extracted with Triton X-114 to separate the detergent soluble 'integral' and water soluble 'peripheral' membrane proteins (IMP & PMP respectively). The distinction between protein profiles of cytosol, membrane and its subfractions was apparent from SDS-PAGE. Serological specificity of IMP and PMP was evidenced by immunoblotting with polyclonal antisera (raised in rabbits) which also served to highlight major seroreactive antigens of both protein pools. A modified silver staining protocol was used to detect the presence of immunosuppressive moiety lipoarabinomannan (LAM) in various subfractions.

A panel of 32 monoclonal antibodies (moAbs provided by WHO-IMMYC) against shared epitopes of 17 mycobacterial antigens, including LAM, was used in ELISA for the antigenic definition of plasma membrane proteins of BCG. The previously described 19,20,33/36 (proline rich antigen) and 38 kDa (Pst-S homolog) proteins were found to be a part of the IMP pool whereas the 14/16 kDa (α-crystallin heat-shock protein, hsp) protein was present in PMP. In addition, the 40 kDa antigen partitioned in both the fractions with a preference for the PMP pool. The 65 kDa hsp

was detected marginally in whole membrane only. Some important antigens which were absent in membrane or its subfractions were : 10,18 and 70 kDa hsps; 23/28 kDa superoxide dismutase, and ~ 30 kDa antigen 85. An interesting picture emerged using moAbs against two distinct epitopes of LAM. The epitope corresponding to ML34 was seen exclusively in the detergent soluble portion of LAM (alongwith IMP pool) whereas mc4311 epitope was noticed mainly in the water soluble LAM (alongwith PMP pool). Modified silver staining of gels could detect LAM only in the IMP pool.

The immunodominance of IMP for human T cell was evident from lymphocyte proliferation assays using BCG membrane, IMP and PMP. In almost all the 20 subjects (comprising tuberculoid leprosy patients and healthy contacts), IMP provided significantly greater activation.

A "continuous elution SDS-PAGE" protocol (developed in our laboratory) for fractionation of IMPs and their subsequent screening by human T cell proliferation assays led to the identification of 3 immunodominant regions in the average molecular weight ranges of : 16-17, ~ 22 and 30-33 kDa, of which the ~ 22 kDa protein(s) induced response in all test subjects. Remarkably, in 4 out of 5 cases, this response was >50% of that observed with whole unfractionated IMP. Presence of LAM in these IMP fractions was monitored by ELISA using the moAb ML34. Fractions in molecular weight range of 40-45 kDa showed positivity for LAM.

Previous reports on mycobacterial membrane proteins were limited only to the description of water soluble ones (PMP) of M.tuberculosis and M.leprae. Contamination of IMP with LAM was considered a major obstacle in the resolution and further characterisation of the former. Having established the immunodominance of IMPs using M.fortuitum as a model system (in which LAM was not a significant constituent of IMP), we were encouraged to work with BCG.

In the present study, despite the presence of LAM, BCG-IMPs have emerged as most potent stimulators of human T cells. The immunodominant IMP subunits of BCG

were devoid of previously characterised immunodominant antigens. Whereas further characterisation of these novel proteins could also reveal the identity of some of them as vital enzymes for exploitation as drug targets, their immunodominance for human T cells definitely makes them attractive candidates for designing future vaccine/ immunotherapeutic agents.

BIBLIOGRAPHY

Abou-Zeid C, Filley E, Steele J, Rook GAW. *A simple new method for using antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocytes in vitro after converting bands cut from western blots into antigen bearing particle.* J Immunol Methods (1987) **98**: 5.

Abou-Zeid C, Geres MP, Inwald J, et al. *Induction of a type 1 immune response to a recombinant antigen from Mycobacterium tuberculosis expressed in Mycobacterium vaccae.* Infect Immun (1997) **65**: 1856.

Akins DR, Purcell BK, Mitra MM, et al. *Lipid modification of the 17 kilodalton membrane immunogen of Treponema pallidum determines macrophage activation as well as amphiphilicity.* Infect Immun (1993) **61**: 1202.

Andersen AB, Andersen P, Ljungqvist L. *Structure and function of a 40,000-molecular weight protein antigen of Mycobacterium tuberculosis.* Infect Immun (1992) **60**: 2317.

Andersen AB, Ljungqvist L, Haslov K, Bentzon MW. *MPB64 possesses 'tuberculosis complex'-specific B-and T -cell epitopes.* Scand J Immunol (1991) **34**: 365.

Andersen AB, Lingqvist L, Oslen M. *Evidence that protein antigen b of Mycobacterium tuberculosis is involved in phosphate metabolism.* J Gen Microbiol (1990) **136**: 477.

Anderson P, Heron I. *Simultaneous electroelution of whole SDS- polyacrylamide gels for the direct cellular analysis of complex protein mixtures.* J Immunol Methods (1993) **161**: 29.

Annonymous. *Serological tests for leprosy.* Lancet (1986) **ii**: 533.

Arand M, Friedberg T, Oesch F. *Colorimetric quantitation of trace amounts of sodium lauryl sulfate in the presence of nucleic acids and proteins.* Anal Biochem (1992) **207**: 73.

Asano A, Cohen NS, Baker RF, Brodie AF. *Orientation of the cell membrane in ghosts and electron transport particles of Mycobacterium phlei.* J Biol Chem (1973) **248**: 3386.

Barksdale L, Kim K-S. *Mycobacterium.* Bacteriol Rev (1977) **41**: 2217.

Barnes PF, Mehra V, Hirchfield SJ, et al. *Characterization of T cell antigen associated with cell wall proteins peptidoglycan complex of Mycobacterium tuberculosis.* J Immunol (1989) **143**: 2656.

Bassey EOE, Catty D, Kumararatne DS, Raykundalia C. *Candidate antigens for improved Serodiagnosis of tuberculosis.* Tubercle and Lung Disease (1996) **77**: 136.

Beckman EM, Porcelli SA, Morita CT, et al. *Recognition of a lipid antigen by CD1-restricted T cells.* Nature (London) (1994) **372**: 691.

Belanger AE, Besra GS, Ford ME, et al. *The embAB genes of Mycobacterium avium encode as arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol*. Proc Nat Acad Sci USA (1996) **93**: 11919.

Berman JS, Blumenthal RL, Kornfeld H, et al. *Chemotactic activity of mycobacterial lipoarabinomannans for human blood T lymphocytes in vitro*. J Immunol (1996) **156**: 3828.

Besra G, Sievert T, Lee R, et al. *Identification of the apparent carrier in mycolic acid synthesis*. Proc Natl Acad Sci USA (1994) **91**: 12735.

Bisht D, Mehrotra J, Dhindsa MS, et al. *A major T cell inducing cytosolic 23kDa protein antigen of the vaccine candidate Mycobacterium habana is superoxide dismutase*. Microbiology (1996) **142**: 1375.

Bloom BR, Fine PEM. *The BCG experience: implications for fut-ure vaccine against tuberculosis*. In Tuberculosis. Pathogenesis, Protection and Control (1994) Ed. Bloom BR. pp. 531-58.

Bloom BR, Murray CJ. *Tuberculosis : Commentary on a reemergent killer*. Science (1992) **257**: 1055.

Boesen H, Jensen BN, Wilcke T, Andersen P. *Human T-Cell response to secreted antigen fractions of Mycobacterium tuberculosis*. Infect Immun (1995) **63**: 1491.

Bordier C. *Phase separation of integral membrane proteins in Triton X-114 solution*. J Biol Chem (1981) **256**: 1604.

Boyum A. *Isolation of mononuclear cells and granulocytes from human blood*. Scand J Clin Lab Invest (1968) **21**(suppl): 77.

Brennan PJ, Nikaido H. *The envelope of mycobacteria*. Ann Rev Biochem (1995) **64**: 29.

Brennan PJ, Cho SN, Klaster PR. *Immunodiagnostics, including skin tests*. Int J Lepr (1996) **64**: S58.

Brisson-Noel A, Gicquel B, Lecossier D, et al. *Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples*. Lancet (1989) **ii**: 1069.

Brodie AF, Kalra VK, Lee SH, Cohen NS. *Properties of energy transducing system in different types of membrane preparations from Mycobacterium phlei-preparation, resolution and reconstitution*. Meth Enzymol (1979) **55**: 175.

Chamberlain NR, Brandt ME, Erwin AL, et al. *Major integral membrane protein immunogens of Treponema pallidium are proteolipids*. Infect Immun (1989) **57**: 2872.

Chanteau S, Cartel JL, Celenier RP, et al. *PGL-I antigen and antibody detection in leprosy patients : evolution under chemotherapy*. Int J Lepr (1989) **57**: 735.

Chatterjee D, Roberts AD, Lowell K, et al. *Structural basis of lipoarbinomannan to induce secretion of tumor necrosis factor*. Infect Immun (1992) **60**: 1249.

Chaturvedi V, Sinha S, Girdhar BK, et al. *Association of mycobacterial specific and Mycobacterium leprale specific antibody level with clinical activity in tuberculoid leprosy patients : a comparative study of three serological enzyme immunoassays*. Lepr Rev (1991) **62**: 122.

Cho SN, Fujiwara T, Hunter SW, et al. *Use of an artificial antigen containing the 3,6-di-o-methyl - ß-D-glucopyranosyl epitope for the serodiagnosis of leprosy*. J Infect Dis (1984) **150**: 311.

Closs O, Harboe M, Axelsen NH, et al. *The antigens of M. bovis strain BCG studied by crossed immuno electrophoresis : a reference system*. Scand J Immunol (1980) **12**: 249.

Coates ARM, Hewitt J, Allen BW, et al. *Antigenic diversity of M. tuberculosis and M. bovis by means of monoclonal antibodies*. Lancet (1981) **ii**: 167.

Colditz GA, Brewer TF, Berkey CS, et al. *Efficacy of BCG Vaccine in the prevention of tuberculosis. Meta-analysis of the published literature*. J Am Med Assoc (1994) **271**: 698.

Condos R, Rom MW, Schluger NW. *Treatment of multidrug resistant pulmonary tuberculosis with interferon-γ via aerosol*. Lancet (1997) **349**: 1513.

Convit J, Sampson C, Zuniga M, et al. *Immunoprophylactic trial with combined Mycobacterium leprae/BCG vaccine against leprosy : Preliminary results*. Lancet (1992) **339**: 446.

Daniel TM, Andersen PA. *The isolation by immunoabsorbent affinity chromatography and physicochemical characterisation of Mycobacterium tuberculosis antigen 5*. Am Rev Respir Dis (1978) **117**: 533.

Daniel TM, Cauthen GM, Collins FM, et al. *Improving methods of detecting infected person at risk of developing disease*. In: Future Research in Tuberculosis. Am Rev Respir Dis (1986) **134**: 401.

Daniel TM, Debanne SM. *The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme linked immunosorbent assay*. Am Rev Respir Dis (1987) **135**: 1137.

Daniel TM, Ellner JJ, Todd LS, et al. *Immunobiology and species distribution of Mycobacterium tuberculosis antigen 5*. Infect Immun (1979) **24**: 77.

Daniel TM, Balestrino EA, Balestrino OC, et al. *The tuberculin specificity in humans of Mycobacterium tuberculosis Antigen 5*. Am Rev Respir Dis (1982) **126**: 600.

Deres KH, Schild H, Wiesmuller K-H, et al. *In vivo priming of virus specific cytotoxic T-lymphocytes with synthetic lipopeptide vaccine.* Nature (London) (1989) **342**: 561.

Dockrell HM, Stoker NG, Lee SP, et al. *T cell recognitions of the 18-kilodalton antigen of M. leprae.* Infect Immun (1989) **57**: 1979.

Dolin PJ, Raviglione MC, Kochi A. *Global tuberculosis incidence and mortality drug 1990-2000.* Bull WHO (1994) **72**: 213.

Eisenach KD, Cave MD, Bates JH, Crawford JT. *Polymerase chain reaction amplification of a repetitive DNA sequence for Mycobacterium tuberculosis.* J Infect Dis (1990) **161**: 977.

Emori K, Nagao S, Shigematsu N. *Granuloma formation by muramyl dipeptide associated with branched fatty acids, a structure probably essential for tubercle formation by Mycobacterium tuberculosis.* Infect Immun (1985) **49**: 244.

Enger HD, Workshop participants. *Results of a WHO sponsored workshop on monoclonal antibodies to Mycobactaium leprae.* Infect Immun (1985) **48**: 603.

Enger HD, Workshop participants. *Results of a WHO sponsored workshop to characterise antigens recognised by Mycobacterium specific monoclonal antibodies.* Infect Immun (1986) **51**: 718.

Erwin KT, Sundaresan T, Gyi MM, et al. *BCG Vaccination of children against leprosy fourteen-year findings of the trial in Burma.* Bull WHO (1985) **63**: 1069.

European Commission Cost/STD initiative on European Vaccine Research. *Report of the expert panels.* Vaccine (1996) **14**: 579.

Evans KD, Nakasome AS, Sutherland PA, et al. *Identification of Mycobacterium tuberculosis and Mycobacterium avium-M. intracellulare directly from primary BACTEC cultures by using acridium ester labelled DNA probes.* J Clin Microbiol (1992) **30**: 2427.

Fifis T, Costopoulos C, Radford AJ, et al. *Purification and characterisation of major antigens from a Mycobacterium bovis culture filtrate.* Infect Immun (1991) **59**: 800.

Filley E, Thole JE, Rook GA, et al. *Identification of an antigenic domain on Mycobacterium leprae protein antigen 85B, which is specifically recognised by antibodies from patients from leprosy.* J Infect Dis (1994) **169**: 162.

Fine PEM, Rodrigues LC. *Modren Vaccines: Mycobacterial diseases.* Lancet (1990) **335**: 1016.

Flynn JL, Chan J, Triebold KJ, et al. *An essential role for interferon - gamma in resistance to Mycobacterium tuberculosis infection.* J Exp Med (1993) **178**: 2249.

Flynn JL, Goldstein MM, Triebold KJ, et al. *Major histocompatibility complex class - I resricted T cell are required for resistance to Mycobacterium tuberculosis infection*. Proc Natl Acad Sci USA (1992) **89**: 12013.

Fridovich I. *Superoxide radical and superoxide dismutase*. Acc Chem Res (1972) **5**: 321.

Gelber RH, Brennan PJ, Hunter SW, et al. *Effective vaccination of mice against leprosy bacilli with subunits of Mycobacterium leprae*. Infect Immun (1990) **58**: 711.

Gelber RH, Mehra V, Bloom B, et al. *Vaccination with pure Mycobacterium leprae inhibits M.leprae multiplication in mouse footpads*. Infect Immun (1994) **62**: 4250.

Germain RN. *MHC- dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation*. Cell (1994) **76**: 287.

Germain RN, Margulies DH. *The biochemistry and cellbiology of antigenic processing and presentation*. Ann Rev Immunol (1993) **11**: 403.

Gill HK, Mustafa AS, Godal T. *Induction of delayed type hypersensitivity in human volunteers immunised with a candidate leprosy vaccine consisting of killed Mycobacterium leprae*. Bull WHO (1986) **64**: 121.

Guleria I, Teitelbaum R, McAdam RA, et al. *Auxotrophic vaccines for tuberculosis*. Nature Med (1996) **2**: 334.

Gulle H , Schoel B, Kaufmann, SHE. *Direct bloting with Viable cells of protein mixtures separated by two- dimensional gel electrophoresis*. J Immunol Methods (1990) **133**: 253.

Hammer J, Valsasnini P, Tolba K, et al. *Promiscous and allele specific anchors in HLA - DR binding peptides*. Cell (1993) **74**: 197.

Hance AJ, Grandchamp B, Levy- Frebault V, et al. *Detection and identification of mycobacteria by amplification of mycobacterial DNA*. Mol Microbiol (1989) **3**: 843.

Hansen GA. *Spedalskhadens arsagen (cause of leprosy), reprinted in translation from Norsk Magazin for Laegevidenskaben 4 (1874), p. 76 - 79*. Int J Lepr (1955) **23**: 307.

Harboe M, Wiker HG. *The 38 kDa Protein of Mycobacterium tuberculosis; A review*. J Infect Dis (1992) **166** :874.

Harboe M, Nagai S, Patarroyo ME, et al. *Properties of proteins MPB64, MPB70 and MPB80 Mycobacterium bovis BCG*. Infect Immun (1986) **52**: 293.

Harth G, Lee B-Y, Wang J, et al. *Novel insights into genetics, biochemistry and immunochemistry of the 30 - kilodalton major extracellular protein of Mycobacterium tuberculosis*. Infect Immun (1996) **64**: 3038.

Haslov K, Andersen A, Nagai S, et al. *Guinea pig cellular immune response to proteins secreted by Mycobacterium tuberculosis*. Infect Immun (1995) **63**: 804.

Hewitt J, Coates ARM, Mitchison DA, Ivanyi J. *The use of monoclonal antibodies without purification of antigens in the serodiagnosis of tuberculosis*. J Immunol Meth (1982) **55**: 205.

Hirschfield GR, McNeil M, Brennan PJ. *Peptidoglycan associated polypeptides of Mycobacterium tuberculosis*. J Bacteriol (1990) **172**: 1005.

Hsich CS, Macatonia SE, Tripp CS, et al. *Development of Th1 CD4+ T cells through IL12 produced by Listeria - induced macrophages*. Science (1993) **260**: 547.

Hunter SW, McNeil M, Modlin RL, et al. *Isolation and characterisation of highly immunogenic cell wall associated protein of Mycobacterium leprae*. J Immunol (1989) **142**: 2864.

Hunter SW, Rivoire B, Mehra V, et al. *The major native proteins of leprosy bacillus*. J Biol Chem (1990) **265**: 14065.

Huygen K, Content J, Denis O, et al. *Immunogenicity and protective efficacy of a tuberculosis DNA vaccine*. Nature Med (1996) **2**: 893.

Ivanyi J, Bothamley GH, Jackett PS. *Immunodiagnostic assays for tuberuilosis and leprosy*. Br Med Bull (1988) **44**: 635.

Ivanyi J, Morris JA, Keen M. *Studies with monoclonal antibodies to mycobacteria*. In: Macario AJL, Macario EC, eds. Monoclonal antibodies against bacteria. New York: Academic Press (1985): 59.

Ivanyi J, Sinha S, Aston R, et al. *Definition of species specific and cross-reactive antigenic determinants of M. leprae using monoclonal antibody*. Clin Exp Immunol (1983) **52**: 528.

Jackett PS, Bothamley GH, Batra HV, et al. *Specificity of antibodies to immunodominant antigens in pulmonary tuberculosis*. J Clin Microbiol (1988) **26**: 2313.

Janis EM, Kaufmann SH, Schwartz RH, Pardoll DM. *Activation of T - Cells in the primary immune response to Mycobacteriun tuber culosis*. Science (1989) **244**: 713.

Kaplan JM, Hunter SW, McNeil M, et al. *Immunological significance of Mycobacterium leprae cell walls*. Prc Nat Acad Sci USA (1988) **85**: 1917.

Kartikeyan S, Chaturvedi RM, Deo MG. *The sociocultural dimension in leprosy vaccine trials*. Lepr Rev (1990) **61**: 50.

Kaufmann SHE. *Immunity to intracellular bacteria*. Ann Rev Immunol (1993) **11**: 129.

Khanolkar- Young S, Workshop participants. *Results of the third Immunology of leprosy / Immunology of tuberculosis antimycobacterial monoclonal antibody workshop*. Infect Immun (1992) **60**: 3925.

Khanolkar SR, Mackenzie CD, Lucas SB, et al. *Identification of Mycobacterium leprae antigen in tissue of leprosy patients using monoclonal antibodies*. Int J Lepr (1989) **57**: 652.

Kirchheimer WF, Storrs EE. *Attempts to establish the armadillo (Dasypus novemcinctus Linn) as a model for the study of Leprosy*. Int J Lepr (1971) **39**: 693.

Klaster PR, de Wit YL, Kolk HJ. *An ELISA inhibition -test using monoclonal antibody for the serology of leprosy*. Clin Exp Immunol (1985) **62**: 468.

Koch R. *Die Atiologie der tuberkulose*. Berliner Klin Wochenschr (1882) **15**: 221.

Kochi A, Vareldzis B, Styblo K. *Multidrug resistant tuberculosis and its control*. Res Microbiol (1993) **2**: 104.

Kohne DE. *The use of DNA probes to detect and identify microorganisms*. In B Kleger, D Jungkind, E Hinks and LA Miller (ed), Rapid methods in clinical microbiology. Plenum Press, New York (1989) p11.

Kumar G, Kalra VK, Brodie AF. *Asymmetric distribution of phospholipids in membrane from Mycobacterium phlei*. Arch Biochem Biophys (1979) **198**: 22.

Kusunose E, Ichihara K, Noda Y, Kusunose M. *Superoxide dismutase from Mycobacterium tuberculosis*. J Biochem (1976) **80**: 1343.

Lamemmli UK. *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. Nature (London) (1970) **227**: 680.

Lartigue DJ, Fite GL. *The chemistry of acid- fast reaction*. J Histochem Cytochem (1962) **10**: 611.

Launois P, Niang N'Diaye M, Cartel JL, et al. *Fibronectin-binding antigen 85 and the 10 kDa GroES-related heat shock protein are the predominant Th1 response inducers in leprosy contacts*. Infect Immun (1995) **63**: 88.

Lee B, Hefta SA, Brennan PJ. *Characterisation of the major membrane protein of virulent Mycobacterium tuberculosis*. Infect Immun (1992) **60**: 2066.

Lenzini L, Rotolli P, Rotolli L. *The spectrum of human tuberculosis*. Clin Exp Immunol (1977) **27**: 230.

Leopold K, Fischer W. *Molecular analysis of the lipoglycans of Mycobacterium tuberculosis*. Anal Biochem (1993) **208**: 57.

Malawi Karonga Prevention Trial Group. *Randomised controlled trial of single BCG, repeated BCG or combined BCG and killed Mycobacterium leprae vaccines for prevention of leprosy and tuberculosis in Malawi*. Lancet (1996) **348**: 17.

Markwell MAK, Haas SM, Bieber LL, Tolbert NE. *A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples*. Anal Biochem (1978) **87**: 206.

Mathews R, Scoging A, Rees ADM. *Mycobacterial antigen specific human T cell clones secreting macrophage activating factors*. Immunology (1985) **54**: 17.

Mehra V, Gong J, Iyer D, et al. *Immune response to recombinant mycobacterial proteins in patients with tuberculosis infection and disease*. J Infect Dis (1996) **174**: 43.

Mehra V, Bloom BR, Torigian VK, et al. *Characterisation of Mycobacterium leprae cell wall associated proteins with the use of T lymphocyte clones*. J Immunol (1989) **142**: 2873.

Mehrotra J, Bisht D, Tiwari VD, Sinha S. *Serological distinction of integral plasma membrane proteins as a class of mycobacterial antigens and this relevance for human T cell activation*. Clin Exp Immunol (1995) **102**: 626.

Mehrotra J, Mittal A, Dhindsa MS, Sinha S. *Fractionation of mycobacterial integral membrane proteins by continuous elution SDS - PAGE reveals immunodominance of low molecular weight subunits for human T cells*. Clin Exp Immunol (1997) **109**: 446.

Miller RA, Dissanayake S, Buchanan TM. *Development of an enzyme-linked immunosorbent assay using arabinomannans from Mycobacterium smegmatis: a potentially useful screening test for the diagnosis of incubating leprosy*. Am J Trop Med Hyg (1983) **32**: 555.

Mitsui A, Hirakawa T, Yodoi J. *Reactive oxygen-reducing and protein refolding activities of adult T cell leukemia-derived factor/human thioredoxin*. Biochem Biophys Res Commun (1992) **186**: 1220.

Mohagheghpur N, Munn MW, Gelber RH, Engeleman EG. *Identification of an immunostimulatory protein from Mycobactrerium leprae*. Infect Immun (1990) **58**: 703.

Moreno C, Mehlert A, Lamb J. *The inhibitory effect of mycobac-terial lipoarabinomannan and polysaccharide upon polyclonal and monoclonal human T cell proliferation*. Clin Exp Immunol (1988) **74** : 206.

Mountford AP, Wilson RA. *Fractionation of schistosome antigen by high - performance electrophoretic chromatography and this screening for the ability to induce the lymphocyte activity*. J Immunol Meth (1993) **160**: 237.

Murray PG, Spithill TW, Handman E. *Characterisation of integral membrane proteins of Leishmania major by Triton X-114 fractionation and analysis of vaccinataion effects in mice*. Infect Immun (1989) **57**: 2203.

Mutis T, Cornelisse YE, Ottenhoff THM. *Mycobacteria induce CD4+ T cells that are cytotoxic and display Th1 like cytokine profile : heterogeneity in cytotoxic acitivity and cytokine secretion levels*. Eur J Immunol (1993) **23**: 2189.

Nagao S, Miki T, Tanak A. *Macrophage activation by muramyl dipeptide (MDP) without lymphocyte participation*. Curr Top Microbiol Immunol. (1981) **25**: 41.

Neill MA, Klebanoff SJ. *The effect of phenolicglycolipid-I from Mycobacterium leprae on the antimicrobial activity of human macrophages*. J Exp Med (1988) **167**: 30.

Noordeen SK. *Elimination of leprosy as a public health problem; why the optimism is justified*. Int J Lepr (1995) **63**: 559.

Osborn MJ, Wu HP. *Proteins of the outer membrane of gram- negative bacteria*. Ann Rev Microbiol (1980) **34**: 385.

Oskam L, Hartskeerl R, Hermans CJ, et al. *A 46 kDa integral membrane protein from Mycobacterium leprae resembles a number of bacterial and mammalian membrane transport proteins*. Microbiology (1995) **141**: 1963.

Ottenhoff THM, Converse PJ, Gebre N, et al. *Cell responses to fractionated Mycobacterium leprae antigen in leprosy. The lepromatous non-responder defect can be overcome in vitro by stimulation with fractionated M. leprae components*. Eur J Immunol (1989) **19**: 107.

Ottenhoff THM, Klaster PP, Ivanyi J, et al. *M. leprae -specific protein antigens defined by cloned human helper T cells*. Nature (London) (1986) **319**: 66.

Pessolani MCV, Brennan PJ. *Mycobacterium leprae produces extracellular homologs of the antigen 85 complex*. Infect Immun (1992) **60**: 4452.

Pessolani MCV, Smith DR, Rivoire B, et al. *Purification, characterisation, gene sequencing and significance of bacterioferritin from Mycobacterium leprae*. J Exp Med (1994) **180**: 319.

Plikaytis BB, Gelber RH, Shinnick TM. *Rapid and sensitive detection of Mycobacterium leprae using a nested primer gene amplification assay*. J Clin Microbiol (1990) **28**: 1913.

Ponnighaus JM, Fine PEM, Sterne JAC, et al. *Efficacy of BCG Vaccine against leprosy and tuberculosis in northern Malawi*. Lancet (1992) **339**: 636.

Rees ADM, Faith A, Roman E, Ivanyi J, et al. *The effect of lipoylation on CD4 T cell recognition of the 19,000 MW Mycobacterium tuberculosis antigen*. Immunology (1993) **80**: 407.

Rees RJW. *The significance of lepromin reaction in man*. Progr Allergy (1964) **8**: 224.

Ridley DS, Jopling WH. *Classification of leprosy according to immunity : a five group system*. Int J lepr (1966) **34**: 255.

Rinke de Wit TF, Bekelie S, Osland A, et al. *The Mycobacterium leprae antigen 85 complex gene family: Identification of the genes for the 85A, 85C and related MPT51 proteins.* Infect Immun (1993) **61**: 3642.

Roche PW, Prestige RL, Watson JD, Britton WJ. *Antibody responses to the 18 kDa protein of Mycobacterium leprae in leprosy and tuberculosis patients.* Int J Lepr (1992) **60**: 201.

Rodrigues LC, Diwan VK, Wheeler JG. *Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a meta-analysis.* Int J Epidemiol (1993) **22**: 1154.

Rogers HJ, Perkins HR, Ward JB. *Microbial cell wall and membranes.* Chapman and Hall, London (1980) p105.

Romain F, Augier J, Pesher P, Marchal G. *Isolation of a proline rich mycobacterial protein eliciting delayed type hypersensitivity reaction only in guinea pigs immunised with living mycobacterium.* Proc Nat Acad Sci USA (1993) **90**: 5322.

Rook GAW. *The Immunology of Leprosy.* Tubercle (1983) **64**: 297.

Salgame P, Abrams JS, Clayberger C, et al. *Differing lymphokine profiles of functional subset of human CD4+ and CD8+ T-cell clones.* Science (1991) **254**: 279.

Sauton B. *Sur La nutrition mnerals du bacille tuberculeux . compte rendu hebdomadaire des seanos de 1'* Academic des Sciences (1912) **155**: 860.

Scott P. *IL-12: initiation cytokine for cell-mediated immunity.* Science (1993) **260**: 496.

Shepard CC. *The experimental disease that follows the injection of human leprosy bacilli into foot-pad of mice.* J Exp Med (1960) **112**: 445.

Shirakava T, Enomoto T, Shimadzu S, Hoppin JM. *The inverse association between tuberculin response and atopic disorder.* Science (1997) **275**: 77.

Sibley LD, Adams LB, Krahenbuhl JL. *Inhibition of interferon-gamma-mediated activation in mouse macrophage treated with lipoarabinomannan.* Clin Exp Immunol (1990) **80**: 141.

Siebert FB, Glen JT. *Tuberculin purified protein derivative. Preparation and analysis of large quantity for standard.* Am Rev Tuber (1941) **44**: 9.

Silva MT, Macedo PM. *The interpretation of the ultrastructure of mycobacterial cell in transmission elecrtron microscopy of ultrathin sections.* Int J Lepr (1983) **51**: 225.

Sinha S, Sengupta U, Ramu G, Ivanyi J. *A serological test for leprosy based on competitive inhibition of monoclonal antibody binding to the MY2a determinant of Mycobacterium leprae.* Trans R Soc Trop Med Hyg (1983) **62**: 468.

Sinha S, Sreevatsa, Gupta SK, Sengupta U. *A comparative study of immunising and delayed hypersensitivity eliciting antigens of Mycobacterium leprae, M. tuberculosis, M. vaccae and M. bovis (BCG).* Int J Lepr (1987) **55**: 42.

Smythe JA, Coppel RL, Brown GV, et al. *Identification of novel integral membrane proteins of P.falciparum.* Proc Natl Acad Sci USA (1988) **85**: 5195.

Sorensen AL, Nagai S, Hoven G, et al. *Purification and characterisation of a low-molecular mass T cell antigen secreted by Mycobacterium tuberculosis.* Infect Immun (1995) **63**: 1710.

Stanford JL, Grange JM. *The meaning and structure of species as applied to Mycobacteria.* Tubercle (1974) **55**: 143.

Stanford JL, Bahr GM, Rook GAW, et al. *Immunotherapy with Mycobacterium vaccae as an adjunct to chemotherapy in the treatment of pulmonary tuberculosis.* Tubercle (1990) **71**: 87.

Stanley SJ, Howland C, Stone MM, Sutherland I. *BCG vaccination of children against leprosy in Uganda final results.* J Hyg (1983) **87**: 233.

Stenger S, Mazzaccarro RJ, Uemura K, et al. *Differential effects of cytolytic T cell subsets on intracellular infection.* Science (1997) **276**: 1684.

Stover CK, de la Cruz VF, Fuerst TR, et al. *New use of BCG for recombinant vaccines.* Nature (1991) **351**: 456.

Styblo K, Meijer J. *Impact of BCG Vaccination programmes in children and young adults on the tuberculosis problem.* Tubercle (1976) **57**: 17.

Surcel H-M, Troye-Blomberg M, Paulie S, et al. *Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens.* Immunology (1994) **81**: 171.

Talwar GP, Zaheer SA, Suresh NK, et al. *Immunotheraputic trials with a candidate anti-leprosy vaccine based on Mycobacterium W.* Trop Med parasitol (1990) **41**: 369.

Tascon RE, Colston MJ, Ragno S, et al. *Vaccination against tuberculosis by DNA injection.* Nature Med (1996) **2**: 288.

Thole JER, Schoningh AAM, Janson T, et al. *Molecular and immunological analysis of a fibronectin-binding protein antigen secreted by Mycobacterium leprae.* Mol Microbiol (1992) **6**: 153.

Thole JER, Stabel LFEM, Suykerbuyk MEG, et al. *A major immunogenic 36,000 molecular weight antigen for Mycobacterium leprae contains an immunoreacrtive region of proline rich repeats.* Infect Immun (1990) **58**: 80.

Thole JER, Wieles V, Clark-Curtiss JE, Ottenhoff THM. *Immunological and functional characterisation of Mycobacteruim leprae protien antigens: an overview.* Mol Microbiol (1995) **18**: 791.

Towbin H, Staehelin T, Gordon J. *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications.* Proc Nalt Acad Sci USA (1979) **76**: 4350.

Triccas JA, Roche PW, Winter N, et al. *A 35-kilodalton Protein is a major target of the human immune response to Mycobacterium leprae.* Infect Immun (1996) **64**: 5171.

Tsai CM, Frasch CM. *A sensitive silver stain for detecting lipopolysaccharides in PAGE.* Anal Biochem (1982) **119**: 115.

Vachula M, Holzer TJ, Kizlaitis L, Andersen BR. *Effect of Mycobacterium leprae phenolic glycolipid-I on interferon gamma augmentation of monocyte oxidative response.* Int J Lepr (1990) **58**: 342-346.

Vega-Lopez F, Brooks LA, Dockrell HM, et al. *Sequence and immunological characterisation of a serine-rich antigen from Mycobacterium leprae.* Infect Immun (1993) **61**: 2145.

Verbon A, Weverling GJ, Kuijper S, et al. *Evaluation of different test for the serodiagnosis of tuberculosis and the use of likelihood ratios in serology.* Am Rev Respir Dis (1993) **148**: 378.

Vordermier HM, Harris DP, Lathigra P, et al. *Recognition of peptides epitopes of the 16,000 MW antigen of Mycobacterium tuberculosis by murine T cells.* Immunology (1993) **80**: 6.

Waters MFR. *The treatment of leprosy.* Tubercle (1983) **64**: 221.

Wessel D, Flugge UI. *A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids.* Anal Biochem (1984) **138**: 141.

WHO Expert Committee on Leprosy. *Sixth Report.* Tech Rep ser (1988) No. **768**.

Wieles B, Van Soolingen D, Holmgren A, et al. *Unique gene organisation of thioredoxin and thioredoxin reductase in Mycobacterium leprae.* Mol Microbiol (1995) **16**: 921.

Wiker HG, Harboe M. *The antigen 85 complex: a major secretion product of Mycobacterium tuberculosis.* Microbiol Rev (1992) **56**: 648.

Wilcke JTR, Jensen BN, Ravn P, et al. *Clinical evaluation of MPT-64 and MPT-59, two proteins secreted from Mycobacterium tuberculosis, for skin test reagents.* Tubercle and Lung Disease (1996) **77**: 250.

World Health Organisation. *Weekly Epidemiological Record.* (1995) **26**: 185.

World Health Organisation. *Report of the study group on chemotherapy of leprosy for control programs.* Tech Rep Ser (1982) **675**.

Wray W, Boulikas T, Wray VP, Hancock R. *Silver staining of proteins in polyacrylamide gels.* Anal Biochem (1981) **118**: 197.

Young DB, Lamb JR. *T lymphocytes respond to solid phase antigen, a novel approach to the molecular analysis of cellular immunity*. Immunology (1986) **59**: 167.

Young D, Kent L, Rees A, Lamb J, Ivanyi J. *Immunological activity of a 38-kilodalton protien purified from Mycobacterium tuberculosis*. Infect Immun (1986) **54**: 177.

Young DB, Duncan K. *Prospects for new intervention in treatment and prevention of mycobacterial diseases*. Ann Rev Microbiol (1995) **49**: 641.

Young DB, Garbe TR. *Lipoprotein antigens of M. tuberculosis*. Res Microbiol (1991) **142**: 55.

Young DB, Fohn MJ, Khanolkar SR, Buchanan TM. *Monoclonal antibodies to a 28 kDa protein antigen of Mycobacterium leprae*. Clin Exp Immunol (1985) **60**: 546.

Young DB, Kaufmann SHE, Hermans PWM, Thole JER. *Mycobacterial Protein antigens: a compilation*. Mol Microbiol (1992) **6**: 133.

Young DB. *Structure of mycobacterial antigens*. Brit Med J (1988) **44**: 562.

Young RA. *Stess proteins and immunology*. Ann Rev Immunol (1990) **8**: 401.

The contribution of previously described immunodominant antigens (19, 33/36and 38 kDa) towards immunodominance of BCG-IMP needs to be considered. Two of them (19 kDa and 38 kDa) were not detectable in M.fortuitum IMP which was found to induce strong T cell activity (Mehrotra et al., 1995) comparable to what is observed with BCG. However, the fact that the three IMPs (19 kDa, 33/36 kDa, 38 kDa) are also 'secretory' (Young and Garbe, 1991; Romain et al., 1993) raises an interesting possibility that the observed immunodominance of secretory antigens (Boesen et al., 1995) could atleast partly be due to the proteins overlapping with IMPs. The extent of such overlap needs to be explored. However, it appears to be only partial since one of the most immunogenic secretory proteins, the 30/31 kDa antigen 85, could not be detected in the BCG membrane; though its presence in the cell wall (Harth et al., 1996) was evident from ELISA using the moAb HYT 27.