

**STUDY OF HELPER T CELL PARAMETERS IN CLINICAL
TUBERCULOSIS**

**THESIS SUBMITTED
UNIVERSITY OF BOMBAY**

**FOR THE DEGREE OF
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IN
MICROBIOLOGY**

**SUBMITTED BY
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**UNDER THE GUIDANCE OF
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***A Thesis
submitted to the
University of Bombay
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Master of Science
in
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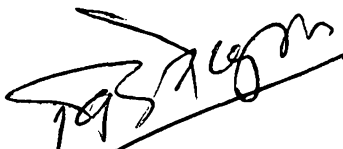
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Certified that the above statement made by the student is correct.



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Signature of the research Guide

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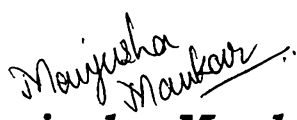
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CONTENTS

• Introduction	1
• Review of Literature	6
• Aims & Objectives	39
• Materials & Methods	47
• Results	62
• Discussion	71
• Summary & Conclusions	83
• Appendix I	86
• Appendix II	89
• References	96
• Synopsis	1 - 11

INTRODUCTION

Tuberculosis is an ancient disease, known to mankind at least for the last 6000 years. It has been for many centuries the most important of human diseases in its global prevalence, dreadful morbidity & massive mortality.

Several investigators (1-4) have studied the serological responses of healthy individuals and patients against different antigens of *M. tuberculosis*. Similarly T cell responses such as tuberculin test & *in-vitro* proliferative response of peripheral blood mononuclear cells (PBMC) against antigens of the organism have also been studied (5-8). Based on these studies investigators have claimed potential utility of such investigations in the laboratory diagnosis of pulmonary and extra pulmonary tuberculosis. However, such claims have been questioned (9). There are usually two types of difficulties in proving the utility of these tests in diagnosis. Firstly, often the contacts, and even the normal healthy individuals without an obvious contact with a case of tuberculosis, reveal significant

responses (1-3,5,8). Secondly, in different reported studies the frequency of positive responses have varied from 10% - 85%, implying that not all patients suffering from overt illness reveal the responses to one or more antigens of the organism used in the assays. Because of this, the feasibility of laboratory diagnosis based on serological or T cell responses has been questioned (9).

Most of the studies referred above, fail to take into consideration the fact that the antibacterial T-helper (Th) responses of the patients suffering from tuberculosis are spectral in nature. This is reported almost twenty years ago by Lenzini *et al* (6). We know that this is due to polar differentiation of Th cells to Th1 or Th2 type. The disease with Th1 type immunity would reveal a strong tuberculin and *in-vitro* antigen specific proliferative response of PBMC and a low antibody titer. In contrast the patients with Th2 type immunity would reveal exactly the opposite. Thus it would be incorrect to expect all the three responses uniformly strong in all patients. Therefore, unless one is familiar with nature of different parameters of Th responses in

different types of clinical tuberculosis, it is difficult to employ them as diagnostic or prognostic markers.

The nature of Th subsets generated in the responses of patients of tuberculosis have also been studied by several investigators (10-14) and the results are contradictory. Significantly, several studies conclude that Th responses in tuberculosis do not reveal polar differentiation (7,12-14). It is difficult to reconcile the results of these studies with those of Lenzini *et al* (6), which revealed a characteristic reciprocal relationship between serum antibody titers against PPD and the then prevalent assays of cell-mediated immunity, namely tuberculin reaction and leucocyte migration inhibition. Based on the assays employed by Lenzini *et al* , one cannot make any claims regarding Th subset differentiation, none were made by the authors either.

Studies claiming that Th responses in tuberculosis do not show polar differentiation are based on assays of cytokine secretion by Th cell clones developed *in-vitro* and cytokine mRNA expression by the PBMC of patients of tuberculosis and

hence cannot be faulted. However the assays being non quantitative in nature, can only ascertain if the Th responses of a given patient are completely polarized to Th1 or Th2 type, in the absence of a polarized response, the assay read out would indicate a mixed Th effector response. It is possible that the Th responses of patients could consist of a mixture of Th0, Th1, and Th2 effectors and under different circumstances there could be a predominance of either the Th1 or the Th2 component. Such a situation can explain the data of Lenzini *et al.* Such relative predominance of Th1 or Th2 effectors in a mixed Th effector response may not be detected by semi quantitative assays.

With these arguments in mind, the three parameters of Th responses, namely tuberculin test, *in-vitro* proliferative response of PBMC to the sonicate of *M. tuberculosis*, and serum antibody titers against the 65-k-Da antigen of the organism, were investigated in the patients suffering from early minimal pulmonary tuberculosis and two types of isolated extra-pulmonary tuberculosis, namely lymphadenitis and meningitis. Extensive pulmonary tuberculosis and classical miliary

tuberculosis have been shown to be associated with a Th2 type response (6). Therefore these were not studied.

***REVIEW OF
LITERATURE***

The ability of mycobacteria to survive and multiply in the host phagocytes constitutes the hallmark of tuberculosis. Destruction of these facultative intra-cellular parasites needs the production of activated macrophages which are generated during cell-mediated immunity (CMI). Macrophages, phagocytic cells - the first line of internal defense respond immediately without specificity. Hence innate immunity also plays a vital role in the pathogenesis of tuberculosis. Thus the relevant literature is reviewed to study the various complexities involved in the mycobacterial infection.

INNATE IMMUNE MECHANISM :

Different infectious agents cause markedly different diseases reflecting the disease process by which they damage tissues. Many extra-cellular pathogens cause disease by releasing specific toxic products or toxins. Intracellular infectious agents frequently cause disease by damaging the cells that house them. When pathogens cross an epithelial barrier, they are thus immediately recognised by phagocytes in the subepithelial connective tissues. The first is the trapping, engulfment and destruction of pathogen by tissue macrophages and migrating

neutrophils, thereby providing an immediate innate cellular immune response.

PHAGOCYTES : The specialized phagocytic cells include blood monocytes, neutrophils and tissue macrophages. Their function is to engulf particles, including infectious agents, internalize them and destroy. The circulating monocytes and tissue macrophages of mammals mediate many of the innate, non-adaptive immune responses to microbial infection. Phagocytic cells have receptors for C3b component of the complement and will more readily phagocytose antigen that has been coated with C3b. Macrophages can also bind pathogens directly. Janeway has proposed that activation of the innate immune system when pathogens bind to non-clonally distributed pattern recognition receptors on cells comprising the innate immune system (15). It was suggested that their receptors exhibit binding specificities for structural patterns typically displayed by cell surface molecules of many organisms such as LPS and glucan, but not normally found on the surfaces of host cells. The ability to mediate pathogen binding should enable pattern recognition receptors to participate both, in the activation of

innate immune cells and in the effector mechanisms (e.g. phagocytosis) that these cell employ.

MACROPHAGES: Macrophages are active phagocytic cells capable of ingesting and digesting exogenous antigens, such as whole microorganisms , insoluble particles , injured or dead host cells and cellular debris. Macrophages mature continuously from circulating monocytes and leave the circulation to migrate into tissues throughout the body. They are found in especially large numbers in connective tissue, in association with gastrointestinal tract, and along certain blood vessels in the liver (Kupffer cells), lung (both interstitial and alveolar) and spleen. Following adherence of the particle to the macrophage membrane, membrane protrusions extend around the attached material. The material is then contained within a membrane bound structure called phagosome which enters the endosomal processing pathway. In this pathway, the phagosome fuses with the lysosome to form a phagolysosome. Once an organism has been internalised, it is exposed to an array of killing mechanisms.

Oxygen Dependent Killing Mechanisms :

Reactive oxygen intermediates (ROIs) : During phagocytosis , a metabolic process known as respiratory burst occurs , which results in the activation of a phagocyte membrane-bound oxidase , reducing oxygen (O_2) to superoxide anion (O_2^-) an ROI, toxic to the pathogens (16). The superoxide anion then gives rise to hydroxyl radicals (OH), singlet oxygen (O) and hydrogen peroxide (H_2O_2) all of which are potentially toxic. The combination of peroxide, myeloperoxide and halide ions constitute a potent halogenating system and additional toxic oxidants such as hypohalite (HIO, HClO) are generated.

Reactive nitric oxide intermediates (RNOIs) : In this pathway nitric oxide synthetase combines oxygen with the guanidino nitrogen and L-arginine to give nitric oxide and citrulline. Studies by Chan *et al* implicate nitric oxide metabolites in host defenses against *Mycobacterium tuberculosis* (17). Nitric oxide can inhibit the secretion of IL-2 and IFN- γ , thus exerting self regulatory effect on Th1 cells which are implicated in immunopathology. Nitric oxide is toxic for bacteria and tumor cells.

Oxygen Independent Killing Mechanisms :

These include a) lysozymes such as muramidase, b) lactoferrin, c) hydrolytic enzymes such as β -glycerophosphatase, d) cationic proteins such as phagocytin, leukin and cathepsin G (18). During phagocytosis, following lysosome fusion there is a transient rise in pH before acidification of the phagolysosome takes place. This pH is optimal for the activity of defensins (19), which are cysteine and arginine rich cationic peptides. They form ion permeable channels in lipid bilayers and act on organisms as diverse as gram positive bacteria, fungi and enveloped viruses.

POLY MORPHO NEUTROPHILS (PMNs) :

PMNs constitute majority of the blood leucocytes. PMNs do not show any inherent specificity for antigens, but they play an important role in acute inflammation together with antibodies and complement, in protection against microorganisms. Their predominant role is phagocytosis. Neutrophils possess two main types of granules. The primary granules are lysosomes containing

acid hydrolases; myeloperoxidase and muramidase (lysozyme), while secondary granules contain lactoferrin in addition to lysozyme and are responsible for bacterial killing.

NATURAL KILLER (NK) CELLS :

NK cells account for upto 15% of the blood lymphocytes. These cells have the capacity to recognize the surface changes that occur on a variety of tumor cells and also on virally infected cells. They are cytotoxic in their action on the above mentioned targets and also to targets coated with IgG antibodies. The latter property is referred to as ADCC (Antibody dependant cell cytotoxicity) and is mediated through the receptor for IgG (Fc γ RIII/CD16). Recent studies have identified cell surface molecules that appear to play important roles in NK cell specificity for their targets. NK cells display activation receptors such as NK R-P1, which is expressed by all murine NK cells that may activate cytotoxicity by transducing biochemical signals (20). A key aspect of NK cell activity is that MHC class I molecules expressed by target cells often result in the inhibition of NK cell mediated lysis (21). Once an NK cell adheres to a target cell, degranulation of perforin-containing

granules occurs. The perforin monomers insert into the target cell membrane forming cylindrical pores which facilitate the entry of the lytic enzymes into the target cell. The activity of NK cells to kill sensitive targets is increased by between 20- and 100- fold when NK cells are exposed to IFN- α and IFN- β or to the NK cell activating factor IL-12, which is one of the cytokines produced early in many infections. IL-12 in synergy with TNF- α , can also elicit production of large amounts of IFN- γ by NK cells, and this secreted IFN- γ is crucial in controlling some infections before T cells have been activated to produce this cytokine. Studies using a variety of experimental models implicate NK cells as important contributors to the innate immune response following infection of the naive host. The ability to secrete TNF- α , GM-CSF and other cytokines also provides NK cells with the potential to activate neutrophils and regulate haemopoiesis (22).

$\gamma\delta$ T CELLS :

Recent evidence demonstrates the important role played by $\gamma\delta$ T cells in resistance to infection. T lymphocytes that express a $\gamma\delta$ T cell antigen receptor (TCR) are a minority population in the

circulation. It is possible that $\gamma\delta$ cells have a MHC class I and/or class II specificity but that they do not require positive selection . Because of their location, it has been proposed that the $\gamma\delta$ T cells are part of a first line of defense (23). $\gamma\delta$ T cells arrive early and help promote the subsequent $\alpha\beta$ response. Studies by Barnes *et al* supported the concept that $\gamma\delta$ cell reactivity is associated with the expression of resistance to tuberculosis in human (24).

COMPLEMENT :

Complement is a collective term designating a series of plasma proteins whose activation is responsible for certain aspects of the host defense and for many features of the inflammatory response. The complement system is a bridge between the cellular and the humoral immune system because it entails the production of molecules that influence cellular immune mechanisms. Complement molecules circulate in the blood in an inactive form , but activation of the first complement component sets into motion a ripple effect. As each component is activated, it acts in turn on the next component in a precise sequence known as the complement cascade. As cleavage fragments are formed, they interact with one another and

assemble complex enzymes that link the stages of the reaction sequence. Many of the smaller fragments formed have important immunological properties that require tight control that augment the immune response.

Complement molecules have four important activities in the immune response : **1)** cell lysis - the later complement component complexes disrupt biologic membranes , **2)** stimulation of an inflammatory response - the cleavage products of several complement components regulate the activities of cells and tissues through chemotactic and anaphylatoxic fragments , **3)** opsonization -several complement component fragments coat target cells (opsonization) so that the phagocytes, which carry receptors for the complement fragments can phagocytose with ease and **4)** removal of antigen - antibody (immune) complexes - complement fragments reduce tissue damaging immune complexes to small soluble aggregates while in the circulation and facilitate clearance of circulating immune complexes (25).

GENETIC BASIS :

The ability of the host to resist infection with a wide range of viral, bacterial and parasitic pathogens is strongly influenced by genetic factors. Natural resistance to infection with intracellular parasites is controlled by a dominant gene on mouse chromosome 1 called Bcg, Lsh or Ity. Bcg affects the capacity of macrophages to destroy ingested intracellular parasites early during infection.

RNA expression studies have showed that Nramp is a gene expressed exclusively in macrophage populations. Nramp encodes an integral membrane protein that has structural homology with known procaryotic and eucaryotic transport systems, suggesting a macrophage - specific membrane transport function. Susceptibility to infection in Bcg resistant and Bcg sensitive strains tested is associated with transmembrane domain 2 of Nramp (26).

Although the specific genes involved have not been identified, recent studies suggest that the basis for this genetic difference resides in a differential capacity of host macrophage to kill phagocytosed tubercle bacilli.

Reactive nitrogen intermediates in particular nitric oxide and its nitrite (NO_2^-) and nitrate (NO_3^-) derivative play a crucial role in the cytostatic and cytolytic activities of macrophages against a variety of microbial and tumour targets. It may be appropriate to discuss the putative role for Nramp in this pathway.

Natural resistance or susceptibility to infection with mycobacteria such as *M. tuberculosis* and *M. leprae* in humans has been shown to be under genetic control similar to that observed in inbred mice and directed by Bcg (26).

Limitations Of Innate Immune Mechanisms Against Facultative Intracellular Parasites :

Mycobacteria possess a thick lipoidal cell wall which renders the organisms impermeable, protecting them from adverse environments, such as that found within macrophages enabling them to resist natural defence mechanisms of the host such as killing by phagocytic cells. As a result, mycobacteria can multiply extensively within macrophages and therefore they are termed as facultative intracellular parasites.

Inactivation of facultative intracellular parasites needs activated macrophages which are generated by the process of CMI, an adaptive immune mechanism.

FACULTATIVE INTRACELLULAR PARASITES :

For a microbe to become pathogenic it must devise strategies to avoid engulfment by phagocytes. Few organisms can survive inside macrophages, and such organisms are termed as facultative intracellular parasites.

Even though macrophages are well equipped to engulf, kill and degrade invading microorganisms, the facultative intracellular parasites can resist killing after phagocytosis. Intracellular pathogens such as those belonging to the genera *Mycobacterium*, *Salmonella*, *Brucella*, *Listeria* and *Legionella* can survive and replicate within normal macrophages by escaping the normal lysosomal destruction either by preventing a phagosome - lysosome fusion (27) or by existing free in the cytoplasm (28) rather than in phagocytic vesicle or by blocking the oxidative burst. Three distinct

mechanisms have been proposed to account for phagolysosome fusion inhibition:

- I) Bacterial release of polyanionic cell wall component, especially sulphatides (27) .
- II) Cyclic AMP has been suggested to inhibit the lysosomal fusion and release of enzymes into phagosomes. The cAMP may be synthesized by the organisms or the macrophage may be induced to do so (29).
- III) Release of ammonia (30).

Mycobacterium tuberculosis is noteworthy for having evolved mechanisms that allows it to survive and multiply inside macrophages . At least some of this success is thought to be linked to the unusual physicochemical properties of the mycobacterial surface

ACTIVATED MACROPHAGES :

In order to destroy facultative intracellular parasites , the host has to develop a more efficient killing mechanism . It was Lurie (31) who first demonstrated the need for activated macrophages in limiting the growth of such bacteria .

The activated macrophages differ from the naive ones in their morphological , biochemical and functional characteristics : they are larger , they attach more rapidly and avidly to glass and plastic and spread more extensively on the substrate (32) ; they display characteristic undulating plasma membrane (33) ; they manifest an increased rate of speed of phagocytosis (34), they display increased total protein synthesis , increased ATP, increased lysosomal enzymes (35) and increased oxygen consumption (36) . The functional change in activated macrophages manifests itself in their increased ability to destroy pathogens (34,35,36,37) . Most of the killing occurs intracellularly after the pathogen has been enclosed in the phagocytic vacuole , the ingested particle is immediately attacked in a variety of ways, all aimed at killing and digesting the foreign body.

Adaptive immune mechanisms, which enhance the microbicidal potential of macrophages, are important in mycobacterial infection.

ADAPTIVE IMMUNE MECHANISMS :

Adaptive immunity reflects the presence of a functional immune system that is capable of specifically recognizing and selectively eliminating foreign microorganisms and molecules. Adaptive immunity displays specificity, diversity and memory.

Adaptive immune responses are not initiated at the site where a pathogen first establishes a focus of infection. They occur in the organised peripheral lymphoid tissues, such as the lymph nodes, to which the pathogen or its products are transported in the lymph, which is produced continuously by filtration of extracellular fluid from the blood. Pathogens infecting peripheral sites will be trapped in the lymph nodes directly downstream of the site of infection; those that enter the blood will be trapped in the spleen; and pathogens infecting mucosal surfaces will accumulate in the Peyer's patches or tonsils. All these lymphoid organs contain APCs specialized for

capturing antigen and activating T cells. Some of these cells capture antigen at the site of infection and then migrate to the downstream lymph node. Naive T lymphocytes circulate continuously from the blood stream to the lymphoid organs, and back to the blood, making contact with many APCs everyday. This ensures that each naive T cell has a high probability of encountering antigens derived from pathogens at any site of infection.

Antigen receptor engagement triggers the activation of a number of downstream signaling events including phosphorylation of protein kinases. Immune activation involves a T or B cell in the resting G0 state entering cell cycle, proliferating and creating a clone of progressively more differentiating cells as activation proceeds ultimately generating effector cells.

T cells recognize antigen in the form of peptide fragments bound to the molecules encoded in the MHC complex. Activation of naive T cells requires **(a)** recognition of a foreign peptide fragment bound to self MHC molecules, **(b)** the simultaneous delivery of a co-stimulatory signal by a specialized APC. Only professional APCs are able to express both classes of MHC molecules as well as the co-

stimulatory surface molecules that drive the clonal expression of naive T cells and their differentiation into armed effector T cells. T cell activation in response to its antigen requires two signaling events (38). The first signal which confers specificity is mediated by the interaction of the TCR with antigenic peptide fragments presented by self class I or class II molecules encoded by the MHC (39). The second signal which induces clonal expansion is provided by the binding of a co-stimulatory molecule B7 to its receptor on T cells CD28. The best characterised co-stimulatory molecules on APCs are the structurally related glycoproteins B7.1 (CD80) and B7.2 (CD86). B7.1 and B7.2 isoforms are expressed on a variety of APCs such as dendritic cells, Langerhan's cells and monocytes. Antigen or CD40 activated B cells also express B7 isoforms. Ligation of CD28 by B7 molecules or by anti-CD28 antibodies will co-stimulate the growth of naive T cells, while antibodies to the B7 molecules which inhibit their binding to CD28, inhibit T cell responses. In addition to B7, three other molecules expressed on the surface of APC, the adhesion molecules, ICAM-1,

LFA-3 and VCAM-I, have been shown to co-stimulate T cell activation.

Activation induces the resting T cell to enter the cell cycle and culminates in the expression of the high affinity receptor for IL-2 and secretion of IL-2. Once activated, the T cells, already having CD28, express an additional receptor CTLA-4. CTLA-4 resembles CD28 and binds B7 molecules about 20 times more avidly than CD28 and delivers a negative signal to the activated T cell which in turn limits the production of T-cell growth factor IL-2 .

T HELPER CELLS :

There are two subpopulations of T cells - T helper (Th) cells and Tcytotoxic (Tc) cells. The Th cells and Tc cells can be distinguished by their display of one of the two membrane glycoproteins; either CD4 or CD8. T cells displaying CD4 generally function as Th cells, whereas those displaying CD8 generally function as Tc cells. In response to the recognition of an antigen - MHC complex, a Th cell secretes various growth factors known collectively as cytokines or, more specifically, lymphokines. As a Th cell is activated, it becomes

an effector cell secreting various cytokines, which play an important role in activating B cells, Tc cells, macrophages and various other cells that participate in the immune response. Differences in the patterns of cytokines produced by activated Th cells results in the qualitative differences in the type of immune response that develops. The generation of both humoral and cell - mediated immune responses depends on the activation of Th cells. The clonally expanded population of antigen - specific Th cells can now play a role in the activation of the B cells and other T lymphocytes and macrophages, that generate the humoral and cell - mediated responses respectively.

Nearly 25 years ago, researches noted that efficient humoral responses to the majority of antigens require the concerted action of T and B cells. Once a Th cell recognizes a processed antigen displayed by class II MHC molecules on the membrane of a B cell the two cells interact forming a T- B conjugate. After antigen specific activation, increased expression of many B and T cell surface adhesion molecules serves to stabilize T-B cell conjugation (40).

Recent identification of CD40 ligand (CD 154) expressed by T cells has helped to elucidate in molecular terms the critical cell - surface molecule on activated helper T cells that provides help to B cells (41). CD40 plays a central role in proliferation of T cell dependent B cells. During an immune response, B cells fulfill an important role, not only as antibody producing cells but also as APC (42) . CD154 can induce the expression of B7.1 and B7.2 on B cells (43), and upgradation on B cells, dendritic cells and macrophages.

The selection and activation of the proper effector mechanism is of critical importance to the host and is subject to precise and complex regulation. This is achieved by CD4+ T cell subsets which secrete distinct patterns of cytokines and play a major role in the regulation of the effector functions induced against a particular infectious agent (44).

Th CELL SUBSETS :

It is during the initial response of naive CD4 T cells to antigen in the peripheral lymphoid tissues that the differentiation of these cells into the two major classes of CD4 effector T cells occurs. This step, at

which a naive CD4 T cell becomes either an armed Th1 cell or Th2 cell, has a critical impact on the outcome of an adaptive immune response, determining whether it will be dominated by macrophage activation or by antibody production. This is one of the most important events in the induction of adaptive immune response.

Two CD4 Th cell subsets can be distinguished based on differences in their pattern of cytokine production : Th1 and Th2 (45). Th1 cells secrete IL - 2, IFN - γ and TNF - β , while Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. T cells expressing cytokines of both patterns have been called as Th0 cells (44). The difference in the cytokines secreted is thought to reflect different biological functions of these two subsets. The Th1 subset is responsible for classical cell-mediated functions such as DTH and activation of Tc cells, whereas the Th2 subset functions more effectively as a helper for B-cell activation. The Th1 subset may be particularly suited to respond to viral infections and intracellular pathogens because it secretes IL-2 and IFN- γ , which activate Tc cells. The Th2 subset maybe more suited to respond to free living bacteria and helminthic parasites and may mediate allergic

reactions, since IL-4 and IL-5 are known to induce IgE production and eosinophil activation respectively. The *in vivo* outcome of the immune response depends on the relative levels of Th1 - like or Th - 2 like activity. Th1 cytokines are generally elevated in responses to intracellular pathogens, and Th2 cytokines are elevated in allergic diseases and helminthic infections.

The cytokines produced by the Th1 and Th2 subsets exhibit cross - regulation, that is, the cytokines secreted by one subset can block the production and/or activity of the cytokines secreted by the other subset. For instance, IFN- γ (secreted by Th1 subset) preferentially inhibits the proliferation of the Th2 subset, and IL-10 (secreted by the Th2 subset) downregulates secretion of IFN- γ and IL - 2 by the Th1 subset (46). Similarly, IFN- γ and IL - 2 (secreted by the Th1 subset) promote IgG1 and IgE production. On the other hand IL-4 (secreted by the Th2 subsets) promotes production of IgG1 and IgE and suppresses production of IgG2a (46).

Strong evidence suggests that Th1 and Th2 cells do not derive from distinct lineages, but rather develop from the same Th

With these arguments in mind, the following aims and objectives were set up.

cell precursor under the influence of several factors such as antigen, cytokines, etc. (47).

One of the oldest factors known to influence the development of Th cell subsets is antigen dose. Early *in-vitro* studies showed that both low and high amounts of antigen primed for DTH, whereas moderate levels stimulated antibody production (48).

It has been demonstrated that low dose challenge in the murine leishmanial system induces a cell-mediated immune response that leads to resistance to reinfection (48). This is more evident from the observed inhibitory effect of high concentrations of antigen on proliferation of Th1 clones but not Th2 clones (49). This may account for the observations that DTH reactions are favoured by low daily doses of antigen while high antibody responses are favoured by high daily doses of antigen (49).

Another factor that influences the differentiation of CD4 T cells into distinct effector subsets is the amount and exact sequence of the antigenic peptide that initiates the response. Large amount of peptides that achieve a high density on the surface of APCs tend to stimulate Th1 responses, while low density presentation tends to

elicit Th2 responses. Moreover peptides that interact strongly with the TCR tend to stimulate Th1-like responses, while peptides that bind weakly tend to stimulate Th2-like responses.

Most protein antigens that elicit CD4 T cell responses stimulate the production of both Th1 and Th2 responses. This reflects the presence in most proteins of several different peptide sequences that can bind to MHC class II molecules and be presented to T cells. Some of these peptides are likely to bind to MHC class II molecules with high affinity, and consequently may be present at high density on the APC, while others may bind with low affinity and be present only at low density. Naive T cells specific for peptide antigens that have high affinity for MHC molecules are likely therefore to encounter a high density of their ligand, while others may only encounter a low density, and these differences in ligand density may affect the subsequent response of the T cell. Indeed, it can be demonstrated experimentally that some peptides in a protein tend to elicit Th2 responses, while others tend to elicit Th1.

CONNECTION BETWEEN INNATE AND ADAPTIVE IMMUNITY :

The mechanisms of innate resistance and those of adaptive immunity are interdependent and they regulate each other. Membrane protein interactions and soluble factors, such as cytokines, antibodies, complement and their receptors on the cells, represent the elements of communication of the intimate crosstalk between the two branches of resistance against pathogens (50) . Most important , the inflammatory responses of the innate resistance set the stage for T cell differentiation by influencing the cytokine milieu in the infected tissues or in the draining lymph nodes in which the antigen specific T cells migrate and expand in response to the antigenic peptides exposed on the APCs .

Studies in both *in vitro* and *in vivo* systems have shown that the cytokines present during primary stimulation are the most important influence on naive Th cell differentiation (46) . This cytokine environment is largely established early in a response by cells participating in innate immune reactions . IL-12 , is one of the cytokines produced early in many infections. IL-12 in synergy with TNF- α can also elicit production of large amounts of IFN - γ by NK

cells and this secreted $1\text{FN-}\gamma$ is crucial in controlling some infections before T cells have been activated to produce this cytokine. IL-12 also favours a Th1 response . The ability to secrete $\text{TNF} - \alpha$, GM-CSF and other cytokines also provide NK cells with the potential to activate neutrophils and regulate haemopoiesis . It has also become apparent that there is an important bidirectional relationship between NK cells and adaptive immune response . Through their release of $\text{IFN-}\gamma$, when antigen specific T cells are starting to undergo clonal expansion and differentiation , NK cells can influence the critical decision of Th1 v/s Th2 cell subset development . Another potential source of cytokines are $\gamma\delta$ T cells . A recent study has provided evidence that $\gamma\delta$ cells can modulate cytokine production by NK cells (51) . This finding is still not well understood, however TNF production by $\gamma\delta$ T cells is suspected to play an important role in this process . Similarly , $1\text{FN-}\gamma$ production by $\gamma\delta$ T cells can prime macrophages to express large amounts of TNF when exposed to LPS (52). These studies cast $\gamma\delta$ T cells as an important regulatory cell type for adaptive and innate immune responses.

SPECTRAL NATURE

Tuberculosis and Leprosy do not occur as single clinical entities but show a wide spectrum of clinical and immunological manifestations exhibiting different patterns of immune responses to the same pathogenic agent in different individuals .

Ridley and Jopling (53) described the clinical and histopathological spectrum of leprosy according to 5 categories . At one pole of the spectrum , tuberculoid leprosy patients develop a high level of specific cell mediated immunity that ultimately kills and clears the bacilli in the tissues , although there is concomitant immunological damage to the nerves. At the lepromatous pole, patients exhibit selective unresponsiveness to antigens of *M. leprae* and the organisms multiply abundantly in the lesions , often to extraordinary numbers - as many as 10^{10} per gram of tissue. Both these forms namely tuberculoid leprosy (TT) and lepromatous leprosy (LL) are relatively stable , whereas the borderline forms are unstable and without treatment deteriorate towards the more stable polar forms . The border line forms are borderline tuberculoid(BT),

borderline(BB) and borderline lepromatous(BL) going from the tuberculoid to the lepromatous pole of spectrum .

Tuberculoid leprosy can be self healing and typically has low levels of antibodies and good CMI . In contrast to this the disseminated lepromatous form of the disease is associated with high levels of relevant and irrelevant antibodies and poor cellular immune function (54). It has therefore been suggested that cell mediated immune responses are linked to protective immunity in leprosy and that the quality of this CMI determines the position the host finds on the disease spectrum.

Skinsnes (55) was amongst the first to suggest that tuberculosis , like leprosy is characterized by immune spectrum. Tuberculosis develops through an immunological spectrum that ranges from a state of high resistance to an opposite extreme low resistance. Lenzini (6) has reported a spectrum of progressive human tuberculosis on clinical and immunological grounds. Patients fall into one of the four groups consisting of a polar reactive group (RR) associated with a well developed CMI and an opposite unreactive group (UU) in which CMI is absent; and two intermediate

groups, one towards the reactive end (RI) and the other towards the unreactive end (UI). The number of acid fast bacilli in the infected tissues increases towards the unreactive pole as CMI diminishes and this is accompanied by an increase in antibody levels. The RR form is characterized by lymphocytes and epitheloid cells in the lesions. In the UU form, bacteria and lesions spread rapidly in lungs and other organs, thus indicating the importance of CMI in progressive mycobacterial disease such as tuberculosis. The spectral nature of the disease is both in terms of resistance to disease and nature of tissue damage. We now know that the spectral nature of the disease is due to polar differentiation of Th cells to Th1 or Th2 type. The nature of Th subsets generated in the responses of patients of tuberculosis have been studied by several investigators and the results are contradictory.

Studies by some investigators (10,11), based on cytokine spectrum of *M. tuberculosis* reactive CD4⁺ T cell clones prepared from the PBMC of patients of tuberculosis, claim that Th responses in tuberculosis reveal polar differentiation. In contrast studies by others (12,13) have reported a wide spectrum and heterogeneity of

cytokine secretion by such T cell clones, questioning the polar differentiation. In still other studies (14) of *in-vitro* antigen induced spectrum of cytokine mRNA expression by PBMC of patients, conflicting results have been reported.

Complete polar differentiation of Th subsets may not be a common feature in tuberculosis as claimed by several investigators (12-14). Except in diseases such as miliary tuberculosis or extreme fibrocaceous lung disease, the responses may not be polar; instead, they could be a mixture of Th0, Th1 or Th2 components, depending upon the circumstances. Such a situation can explain the data of Lenzini *et al.*

Laboratory Diagnosis Of Tuberculosis Employing The Host Immune Responses:

Serological responses of healthy individuals and patients against different antigens of *Mycobacterium tuberculosis* have been studied by several investigators (1-4). Similarly, T cell responses such as tuberculin test and *in-vitro* proliferative response of PBMC against antigen of the organism have also been studied (5-8). Based

on these studies investigators have claimed potential utility of such investigations in the laboratory diagnosis of pulmonary and extra-pulmonary tuberculosis. However, such claims have been questioned (9). There are usually two types of difficulties in proving the utility of these tests in the diagnosis. Firstly, often the contacts and even the normal healthy individuals without an obvious contact with a case of tuberculosis, reveal significant responses (1-3,5,8). Secondly, in different reported studies, the frequency of positive responses have varied from 10% to 85%, implying that not all patients suffering from overt illness reveal the responses to one or more antigens of the organism used in the assays. Because of this, the feasibility of laboratory diagnosis based on serological and T cell responses has been questioned (9).

Most of the studies referred above fail to take into consideration the spectral nature of the disease that was reported almost 20 years ago by Lenzini *et al* (6). We now know that this is due to polar differentiation of Th cells to Th1 or Th2 type. The nature of the Th subsets generated in the responses of patients of tuberculosis have also been studied by several investigators (10-14)

and the results are contradictory. Significantly, several studies conclude that Th responses in tuberculosis do not reveal polar differentiation (7,12-14). It is difficult to reconcile the results of these studies with those of Lenzini *et al* (6). Studies claiming that Th responses in tuberculosis do not show polar differentiation, are based on assays of cytokine secretion by Th cell clones developed *in vitro* and cytokine mRNA expression by the PBMC of patients of tuberculosis and hence cannot be faulted. However, the assays being non-quantitative or semi-quantitative in nature, can only ascertain if the Th responses of a given patient are completely polarized response, the assay read out would indicate a mixed Th effector response. It is possible that the Th responses of the patients could consist of a mixture of Th0, Th1 and Th2 effectors and under different circumstances there could be a predominance of either the Th1 or the Th2 component. Such a situation can explain the data of Lenzini *et al*. Such relative predominance of Th1 or Th2 effectors in a mixed Th effector response may not be detected by semi-quantitative assays.

AIMS AND OBJECTIVES:

To study the Th responses against antigens of *M. tuberculosis* in normal healthy individuals and patients of tuberculosis.

The following Th responses were studied:

- Response to tuberculin test.
- *In-vitro* proliferation of PBMC induced by sonicate of *M. tuberculosis*.
- Serum antibody titers against 65-k-Da antigen of *M. tuberculosis*.

MATERIALS AND METHODS

1. PATIENTS :

- (a) Pulmonary tuberculosis - Twenty five patients with localized infiltrative lesions with or without cavitation, limited to one or two segments of a lobe only, on chest X ray examination and with acid fast bacilli (AFB) in sputum were included in this study. Five cases had pleural effusion also.

- (b) Tuberculous lymphadenitis - Six patients with enlarged cervical lymph nodes, belonging to a single group were included in this study. Radiologically, these patients did not have any pulmonary or hilar lesions. Lymph node biopsy of all the six cases revealed caseating epitheloid cell granulomata with scanty AFB.

- (c) Tuberculous meningitis- Seven patients with symptoms and signs of acute/subacute meningitis having a cerebro spinal fluid (CSF) cytological and chemical profile suggestive of tuberculous meningitis, were included in this study. AFB were

not seen in the smears of CSF. None of the patients had radiological evidence of pulmonary tuberculosis.

(d) Miliary tuberculosis- A single case of classical miliary tuberculosis was also included for comparison, as a non reactive (UU) case as per classification of Lenzini *et al* (6).

(e) Asymptomatic healthy individuals- Ten healthy adults were also included in the study for comparison.

2. MICROORGANISMS:

Mycobacterium tuberculosis H37Rv (NCTC 7416) was purchased from National Collection of Type Cultures, Collindale, London. The strain was maintained on Lowenstein Jensen's medium slants and cultivated on the Douv and Youmans medium (56) (Appendix I.1.).

3. ANIMAL :

Adult albino rabbits weighing 1.5 - 2.0 Kg were purchased from Haffkine Biopharmaceutical Corporation Ltd., Mumbai and used for raising antisera against the 65-kDa antigen of *M.tuberculosis* .

4. PREPARATION OF ANTIGEN:

(a) Preparation of whole sonicate of *M. tuberculosis* - Five - six week old culture of *M. tuberculosis* in the Douv and Youmans medium was harvested and then subjected to 2.5 Mrad gamma irradiation in a Co⁶⁰ chamber. The irradiated cells were washed with phosphate buffered saline, pH 7.2 (PBS- Appendix I.2.) and subjected to sonic disintegration in a sonicator (Branson sonifier, B-30, 20 kHz, 50% duty cycle) for 45 mins and spun at 50,000g for 1h. The supernatant was filtered through a 0.22 µm membrane filter (Millipore, USA) and its protein content was estimated by the method of Lowry *et al* (57) (Appendix II.a.). It was then aliquoted and stored at -20°C and used as “test antigen”, in lymphoproliferative assays.

(b) Preparation of Lysozyme-Papain digested sonicate - Sonicate of *M. tuberculosis* H37Rv, prepared as described earlier, was treated with Lysozyme (SIGMA) (2mg/ml concentration) and incubated at 37°C water bath for 2h. 10 μ M EDTA was added to the mixture for the enzyme reaction. A small aliquot of the sonicate was removed and spun at 50,000g for 1h and its protein content estimated. The lysozyme treated sonicate was subjected to Papain digestion by adding 1 mg Papain (SIGMA) for 15 mg of protein content in the sonicate. 0.1 M L-cysteine HCl (0.5 ml/30 ml mixture) was added to facilitate the digestion. The digestion was allowed to proceed at 37°C for 2h in the water bath with intermittent shaking. The reaction was then stopped by addition of 0.13 M iodoacetamide in the proportion of 0.2 ml for every 10 ml of reaction mixture and simultaneously transferring the flask to a chilled ice bath. The suspension was centrifuged at 50,000g for 1h at 4°C, supernate collected, its protein content estimated and was then aliquoted and stored at -20 °C.

5. ISOELECTROFOCUSSING PROCEDURES:

- (a) Analytical isoelectrofocussing (IEF) - It was done using a standard procedure (58). A 0.5mm thick polyacrylamide gel was prepared on a gel bond film (6 cm x 12 cm) with 7.5% polyacrylamide in distilled water containing 2% ampholine (SIGMA) of 3.5 - 5.0 pH range. Strips of Whatman filter paper number one, soaked in the appropriate anolyte and catholyte solutions were placed directly on the gel at the respective electrode ends. 0.05 M H_2SO_4 , pH 11.5 was used as anolyte and 0.4 M HEPES, pH 5.2 was used as catholyte. Platinum wire electrodes were placed directly in contact with filter paper strips and prefocussing was carried out on a Multiphor Electrophoresis Unit (LKB) at 5 mA current for 30 min. 10 μl of sample containing 15 mg/ml of protein was applied on a Whatman filter paper number one strip of 5 x 10mm size and kept at the cathodal end of the gel. Focussing was carried out at constant power of 2.5W, maximum voltage of 1200V and maximum current of 5mA, at 4°C for heat dissipation from the gel. After 60 min, pH gradient was checked with a surface pH electrode (LKB, Pharmacia). The gels were immediately

transferred into a fixing solution and kept overnight. Next day they were stained by silver staining technique as described by Blum *et al* (59) (Appendix II.c.)

- (b) Preparative IEF on agarose - Preparative IEF was done by techniques described earlier (58). 20 ml of 1% agarose (SIGMA, IEF grade type VIII, < 0.02 EEO) containing 12.5% (w/v) sucrose and 2% (v/v) Ampholine (SIGMA) of 3.5 - 5.0 pH range was layered onto a gel bond film of 7.5 cm x 7.5 cm size and cooled to solidify. Filter paper strips (Whatman number 17) dipped in the appropriate anolyte and catholyte solutions were placed at either sides of the gel at respective electrode ends. 0.05 M H₂SO₄, pH11.5 was used as anolyte and 0.4 M HEPES, pH5.2 was used as catholyte. Prefocussing was carried out at 12 mA current and 250 V voltage for 30 min. A trough of the size 0.5 cm x 0.6 cm was cut 5mm from the cathodal end and 1 ml of lysozyme - papain digested sonicate of *M. tuberculosis*, containing 15 mg/ml of protein was added in it. Electrofocussing was carried out at constant power of 3 W, keeping maximum current at 12 mA and maximum voltage at 1000 V, for 3h, after which the current

gradually came down. Entire electrofocussing was carried out at 4°C. Distinct bands of precipitated proteins could be identified. pI of the bands on the gel was checked by a surface electrode and pH gradient formation was also confirmed.

6. PREPARATION OF RABBIT ANTIBODY AGAINST 65-k-Da ANTIGEN OF *M. tuberculosis* :

It has been shown earlier that the 65-k-Da antigen of *M. tuberculosis* has a pI of 4.3. The lysozyme-papain digested sonicate of *M. tuberculosis* was subjected to preparative agarose IEF as described above and the visible precipitin band at pI 4.3 was cut. The bands from three gels were pooled together and crushed in a glass tube with glass piston to a fine particle size. To this mixture, equal quantity of Freund's adjuvant (incomplete) (SIGMA) was added and a homogenous emulsion prepared by grinding with a tight fitting glass piston. A rabbit weighing 2.5 Kg was depilated and the antigen preparation was injected intradermally at multiple sites on the back. Immunisation was repeated two more times at 10 days interval. Seven days after the last immunisation the rabbit was test bled from ear

artery, blood collected and the serum separated. The antiserum was tested against *M. tuberculosis* H37Rv sonicate by agarose gel diffusion method of Ouchterlony (60) and the antibody content was titrated by the same method. When a sufficiently high titre was obtained (1:16 or above), the rabbit was completely bled out, serum separated, decomplexed, aliquoted and stored at -20°C. IgG fraction was obtained from this antiserum by Ammonium Sulphate Precipitation (Appendix II. d.).

7. TUBERCULIN TEST:

A standard tuberculin test, an index of delayed type hypersensitivity (DTH), was performed with 0.1 ml containing 10 TU PPD (Span Diagnostics) and erythema and induration was recorded after 48h.

8. LYMPHOPROLIFERATION TEST:

This assay was carried out employing standard microculture technique (61). Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll - hypaque density gradient

centrifugation (Density 1.077+ 0.001 g/ml, SIGMA). Viability of cell suspension was assayed by trypan blue exclusion test. Viable cells were suspended at 2×10^6 cells/ml in the culture medium RPMI 1640, containing 5% Foetal Calf Serum (FCS, Biological Laboratories, Israel), 2mM L - Glutamine (SIGMA) and 0.5% Antibiotic - Antimycotic (SIGMA, final concentration in the culture medium, 50 units/ml Penicillin, 50 ug/ml Streptomycin and 0.012 μ g/ml Amphoterecin B). Each well of the tissue culture plate (Nunc, Denmark, 96U) received 2×10^5 cells and sonicate of *M. tuberculosis* H37Rv (3 μ g/culture). A parallel set of cultures were also stimulated with the T cell mitogen PHA (Phytohaemagglutinin) (SIGMA, 2-10 μ g/ml) in the final volume of 200 μ l/well. Control wells did not contain antigen. Cultures were incubated in a CO₂ incubator (Flow Laboratories) at 37°C and 5% CO₂ in humidified air. 1 μ Ci of 3H labelled thymidine (BARC, India, specific activity 6.5 Ci/mMole) was added 72h later for PHA and 120h later for antigen. After 18h of pulsing, the cultures were harvested on 3mm Whatman filter paper discs, air dried and soaked in 10% trichloroacetic acid for 10 min, followed by three washes of 2min each with distilled water and finally a wash with 20% methanol for 5min.

Discs were dried and transferred into scintillation vials containing 4ml scintillation fluid (Appendix II.9.). Incorporation of the label was assayed in a β -scintillation counter (Rackbeta1211, LKB) and expressed as counts per minute (CPM). Degree of stimulation was expressed as stimulation index (SI), which is the ratio of CPM with to without antigen.

9. ASSAY FOR ANTI 65-k-Da ANTIBODY LEVELS:

Antibody levels against 65-k-Da antigen of *M. tuberculosis* H37Rv in the sera of patients were assayed by indirect sandwich ELISA technique, as described earlier (62).

Design of the assay - ELISA plate wells (Nunc, Denmark, Maxisorp) were coated with rabbit antibody against 65-k-Da antigen to capture the 65-k-Da antigen from *M. tuberculosis* H37Rv sonicate. Test serum samples and appropriate controls were added onto the complex. The anti 65-k-Da antibodies in the test sera were detected by using anti-human IgG specific peroxidase conjugate.

Reagents -

- (a) Coating antibody: IgG fraction of rabbit antiserum against 65-kDa antigen of *M. tuberculosis* H37Rv in coating buffer (Appendix I.4.).
- (b) Source of 65-kDa antigen: Lysozyme - Papain digested sonicate of *M. tuberculosis* H37Rv in PBS.
- (c) Normal human serum.
- (d) Anti - human IgG specific HRP conjugate (SIGMA). This was used at 1:40,000 dilution.
- (e) Substrate solution: OPD - 4mg/10ml, H₂O₂ - 2μl (30%) added to substrate buffer (Appendix I.5.) and used immediately.
- (f) Dilution buffer: PBS - T20 (Appendix I.6.).
- (g) Washing solution: Saline - T20 (Appendix I.7.).

Procedure:

Wells of ELISA plates (NUNC, C12 Maxisorp, Denmark) were coated with 100μl of IgG fraction of rabbit antiserum against 65-kDa antigen. The plates were incubated overnight at 4°C and for half an hour at 37°C the next day. The well contents were discarded, and

well surfaces blocked with 2% gelatin in coating buffer for 1.5h at 37°C. The plates were washed with washing solution on an ELISA washer (Organon, Teknika, miniwasher) and 100µl of Lysozyme - Papain digested sonicate was added to the wells and incubated at 37°C for 2h. The wells were then washed 5-6 times and 100µl of different dilutions of test serum samples of patients along with appropriate controls were added to the wells. The plate was then incubated at 37°C for 2h. The plate was washed 5-6 times and 100µl of anti human HRP conjugate (1:40,000 dilution) was added to the wells and incubated at 37°C for 2h. The plate was again washed and 100µl of substrate solution was added to the wells and incubated at room temperature in the dark for 12-15 min. After colour development, the reaction was stopped with 2.5M HCl and read at 492nm in an ELISA reader (Titretek Multiscan - Flow Laboratory).

Calculations:

$$\text{Antibody titre} = \frac{\text{A492 of test serum sample}}{\text{A492 of normal human serum}} \times \text{dilution of test serum} \times 10$$

The antibody titers were expressed as Absorbance units (492nm) per ml (AU/ml).

RESULTS

The three parameters of Th responses, namely tuberculin test, *in - vitro* proliferation of PBMC induced by the sonicate of *M. tuberculosis* H37Rv and the serum anti 65-k-Da antibody titers, were investigated in normal healthy individuals and the three forms of tuberculosis. Even normal healthy individuals revealed significant responses in all the three parameters studied. The responses of normal individuals were used as reference levels. The patients' responses were considered significant only when they were greater than those given in normal healthy individuals.

Tuberculin Test:

The tuberculin responses of patients as well as normal healthy individuals showed considerable variation. The median responses of the patients were higher than those of the healthy individuals. Half the healthy individuals showed a tuberculin response greater than 10.0 mm in diameter. Tuberculin test, which is a test for DTH, has been considered an index of Th1 type response. The median responses of patients belonging to different groups were comparable (fig 1), irrespective of their *in-vitro* antigen

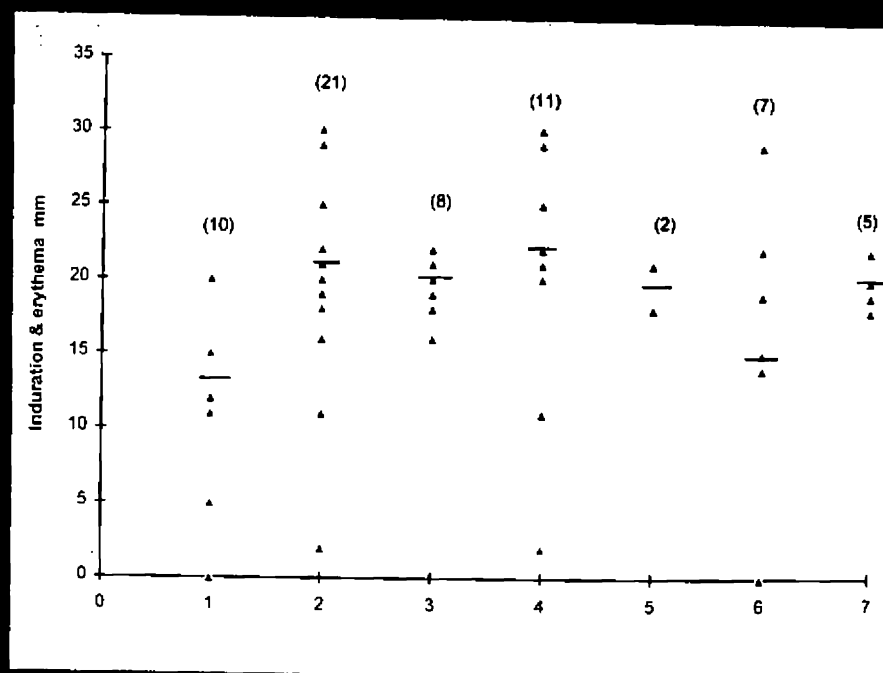


Fig 1. Tuberculin responses of normal healthy adults and patients of tuberculosis.

10 TU of PPD was injected intradermally in the left forearm and erythema and induration read 48 h later.

X-axis - 1) Normal healthy adults; 2) Pulmonary tuberculosis all patients; 3) Pulmonary tuberculosis with Th1 predominant response; 4) Pulmonary tuberculosis with Th2 predominant response; 5) Pulmonary tuberculosis with mixed Th response; 6) Tuberculosis meningitis; 7) Tuberculous lymphadenitis; 8) Miliary tuberculosis.

Horizontal bars indicate the medians.

Figures in parenthesis represent total number of patients.

specific T cell proliferative response (described later), which is also an index of Th1 type immunity. The two responses could not be correlated.

In-vitro antigen specific proliferative response of PBMC of normal healthy adults and patients of tuberculosis:

All six cases of lymph node tuberculosis revealed strong proliferative responses, ranging from 8000-27,000 CPM, with a median of 10,000 CPM, which was 5 times that seen in the healthy individuals. All the cases of tuberculous meningitis showed uniformly poor proliferative responses comparable to those seen in the normal healthy individuals. In pulmonary tuberculosis cases a wide variation was observed (1000-23,000 CPM, with a median of 4500 CPM). Practically half the cases revealed a proliferative response in the same range as that seen in the healthy individuals.

Anti 65-k-Da antibody titers of patients of tuberculosis:

All the six cases of lymph node tuberculosis revealed uniformly low titers , of less than 300 AU/ml, i.e. in the same range

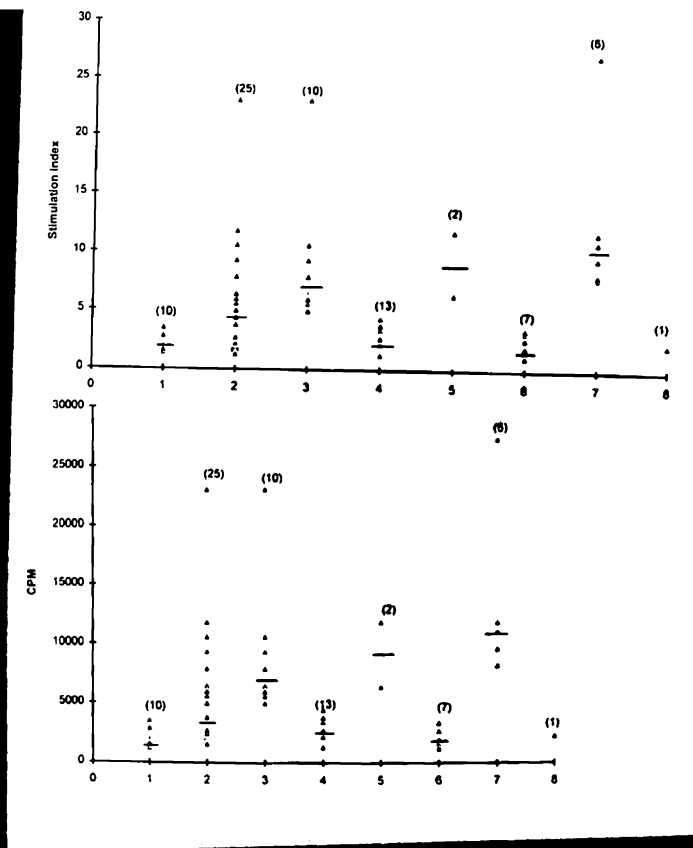


Fig 2. *In-vitro* antigen specific proliferative responses of PBMC of normal healthy adults and patients of tuberculosis.

A standard microculture proliferation assay against the sonicate of *M.tuberculosis* was performed with the PBMC of normal healthy adults and the patients of tuberculosis.

X-axis - same as in Fig 1. Both absolute counts and stimulation indices are shown. CPM- counts per minute.

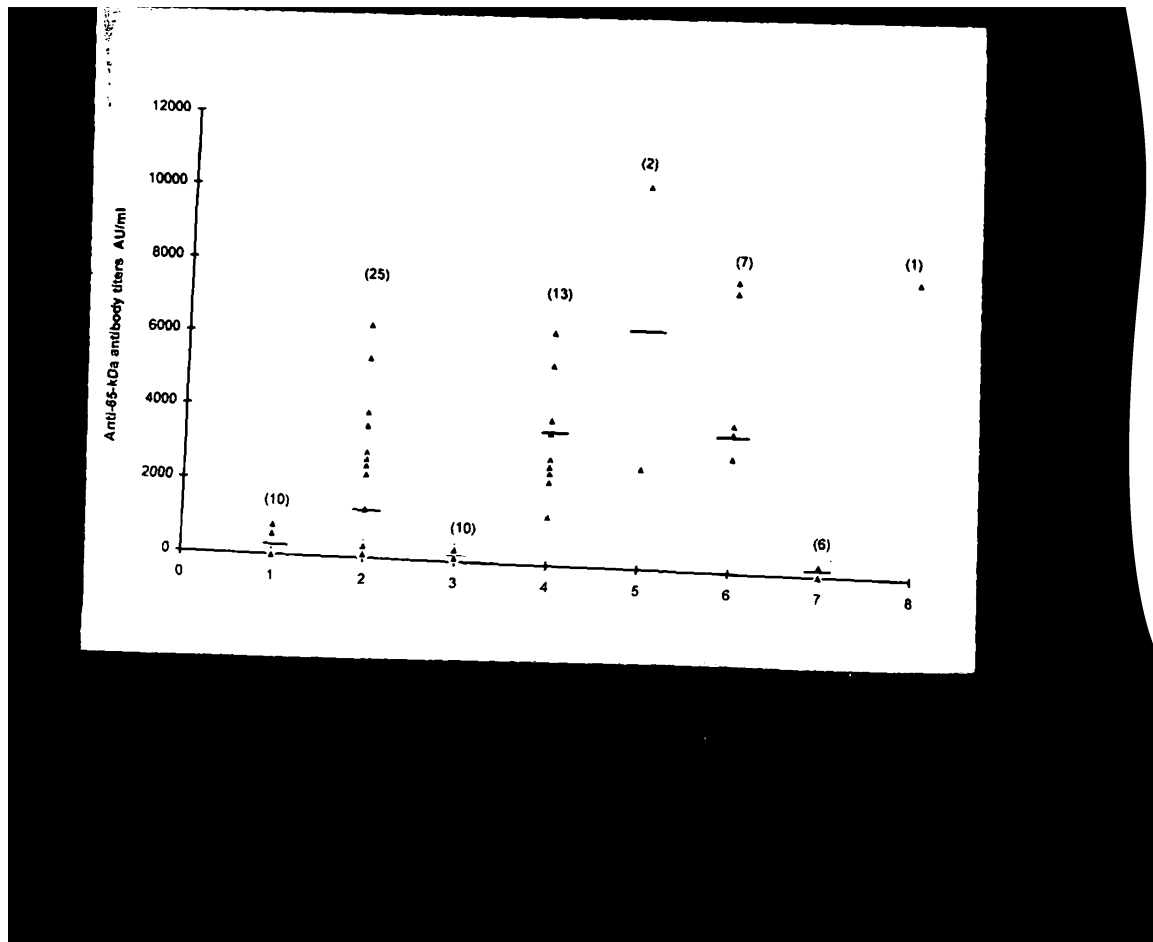


Fig 3. Anti-65-kDa antibody titers of normal healthy adults and patients of tuberculosis.

Serum antibody titers against the 65-kDa antigen of *M.tuberculosis*, were assayed by ELISA and expressed as Absorbance Units (AU) per ml. X- axis - same as in Fig1.

as those of normal healthy individuals. All the cases of tuberculous meningitis revealed very high anti 65-k-Da titers, 2700-8100 AU/ml, with a median titre of 3900 AU/ml, which was 23 times that observed in the normal healthy individuals. The cases of pulmonary tuberculosis showed a wide variation in their serum anti 65-k-Da titers, ranging from zero to 6300 AU/ml with a median titre of 1500 AU/ml.

Healthy individuals:

Even the healthy individuals revealed both *in-vitro* antigen specific proliferation of PBMC and anti 65-k-Da antibody response (figs 2 & 3). More than half the individuals revealed a significant antigen specific proliferative response of 2000-4000 CPM (SI 2-4). The anti 65-k-Da antibody titers varied from practically zero to about 800 AU/ml (fig 3). This data was employed as a basal level of response for comparison with the responses of the patients.

Analysis of relationship between the *in-vitro* antigen specific proliferative responses of PBMC and serum anti 65-k-Da antibody titers of patients of tuberculosis:

The *in-vitro* *M. tuberculosis* proliferative responses of PBMC and the serum anti 65-k-Da titers revealed a reciprocal relationship in 37 out of 39 patients studied. As can be seen in fig 4, whenever *in-vitro* antigen specific proliferative response was strong, the anti 65-k-Da titre was low and *vice-versa*. This was true for all the cases irrespective of the site of disease, as can be seen in figs 5, 6 & 7. In two cases both the responses were elevated. In no patient both the responses were normal. Based on such reciprocal relationship, one cannot unequivocally conclude that the patients had polar Th1 or Th2 type responses, without actually investigating cytokine production by the patients' T cells. However, since a strong *in-vitro* antigen specific proliferative response is indicative of high frequency of IL-2 producing Th cells, one may suggest a higher component of Th1 type effectors in such patients, especially in the light of low anti 65-k-Da titers. Similarly, the reverse situation may be an index of high frequency of IL-4 producing Th cells, implying a

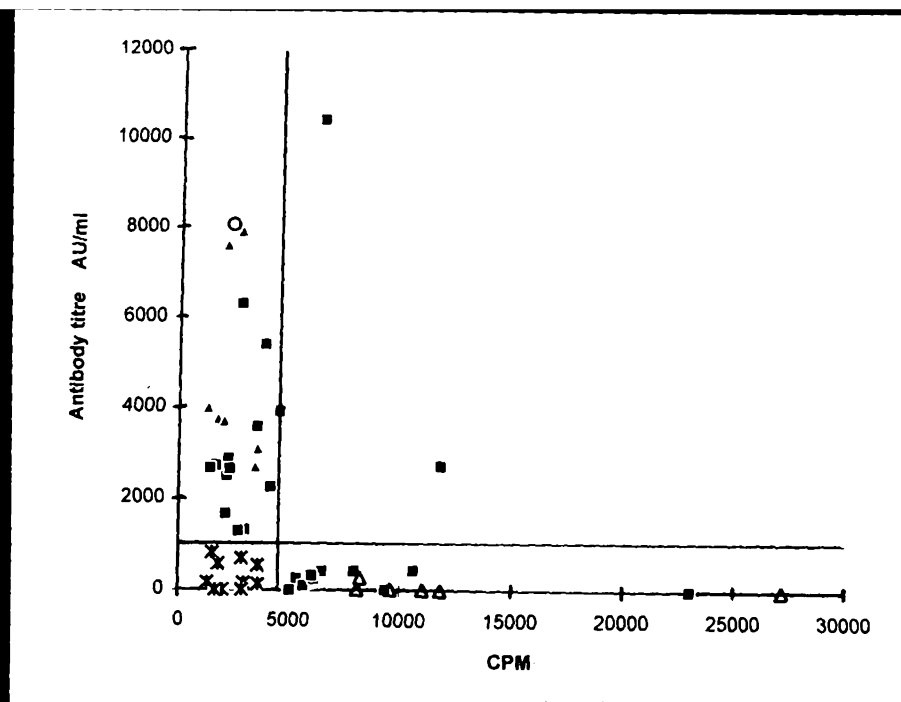


Fig 4. Comparison of the *in-vitro* antigen specific proliferative responses of PBMC and serum anti-65-kDa antibody titers of normal healthy adults and patients of tuberculosis.

X- axis - *In-vitro* antigen specific proliferation.

Y-axis - Anti-65-kDa antibody titer.

(*) Normal healthy adults; (■) Patients of pulmonary tuberculosis;

(▲) Patients of tuberculous meningitis; (Δ) Patients of tuberculous lymphadenitis; (O) The patient of miliary tuberculosis.

The horizontal and vertical lines dividing the scatter gram into four quadrants, indicate mean + 2SD limits of responses of normal healthy adults. Depending upon the two responses, the patients fall into one of the four quadrants.

Lower left quadrant - Normal healthy adults and patients having comparable responses; Upper left quadrant - Patients with a high anti-65-kDa titre and a poor *in-vitro* antigen specific proliferative response ; Lower right quadrant - Patients with a strong *in-vitro* antigen specific proliferative response and a poor anti-65-kDa antibody titer; Upper right quadrant - Patients having both the responses strong.

greater Th2 component in the patients Th responses. When such an analysis was applied to the cases of three types of diseases an interesting relationship between the disease site and Th responses was observed.

All six cases of lymph node tuberculosis which showed strong *in-vitro* antigen specific proliferative responses and poor anti 65-k-Da titers, appeared to have a homogenous Th1 predominant response. In contrast, all seven cases of tuberculous meningitis, who showed a poor *in-vitro* proliferative response against PBMC and a strong antibody response, appeared to have a Th2 predominant response. The analysis of Th responses of patients of pulmonary tuberculosis however revealed heterogenous pattern. It consisted of three groups. One group of ten patients had strong *in-vitro* antigen specific proliferative responses of PBMC, ranging from 5000-23,000 CPM, with a median of 7000 CPM, which was 3.5 times the median response of the healthy individuals. The antibody titers of these patients varied from practically zero to 500 AU/ml, i.e. in the same range as that of the healthy individuals. Thus it was concluded that these ten patients formed a subgroup of

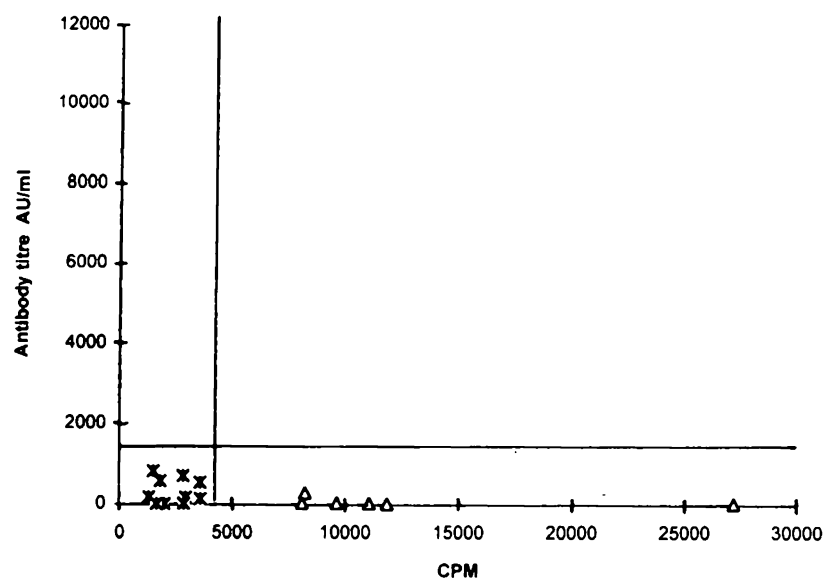


Fig 5. Comparison of the *in-vitro* antigen specific proliferative responses of PBMC and serum anti-65-kDa antibody titers of normal healthy adults and patients of tuberculous lymphadenitis. X-axis and Y-axis - Same as in Fig 4.

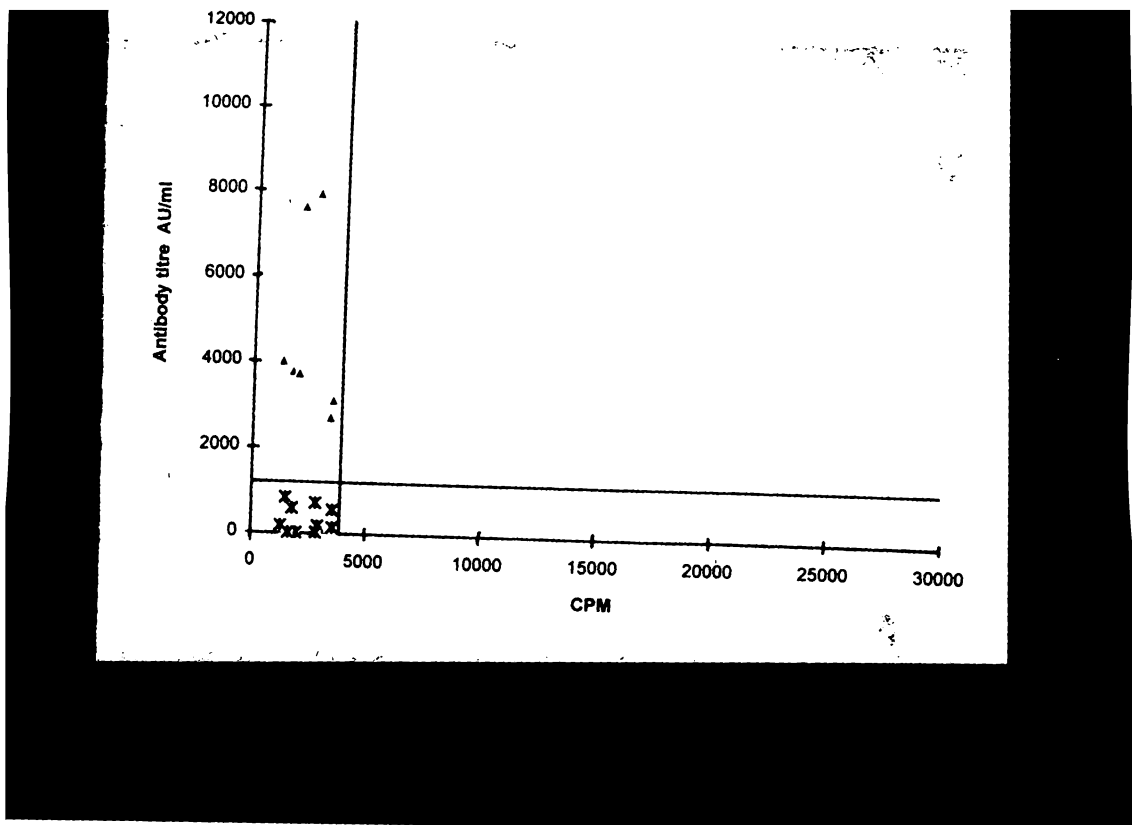


Fig 6. Comparison of the *in-vitro* antigen specific proliferative responses of PBMC and serum anti-65-kDa antibody titers of normal healthy adults and patients of tuberculous meningitis. X-axis and Y-axis - Same as in Fig 4.

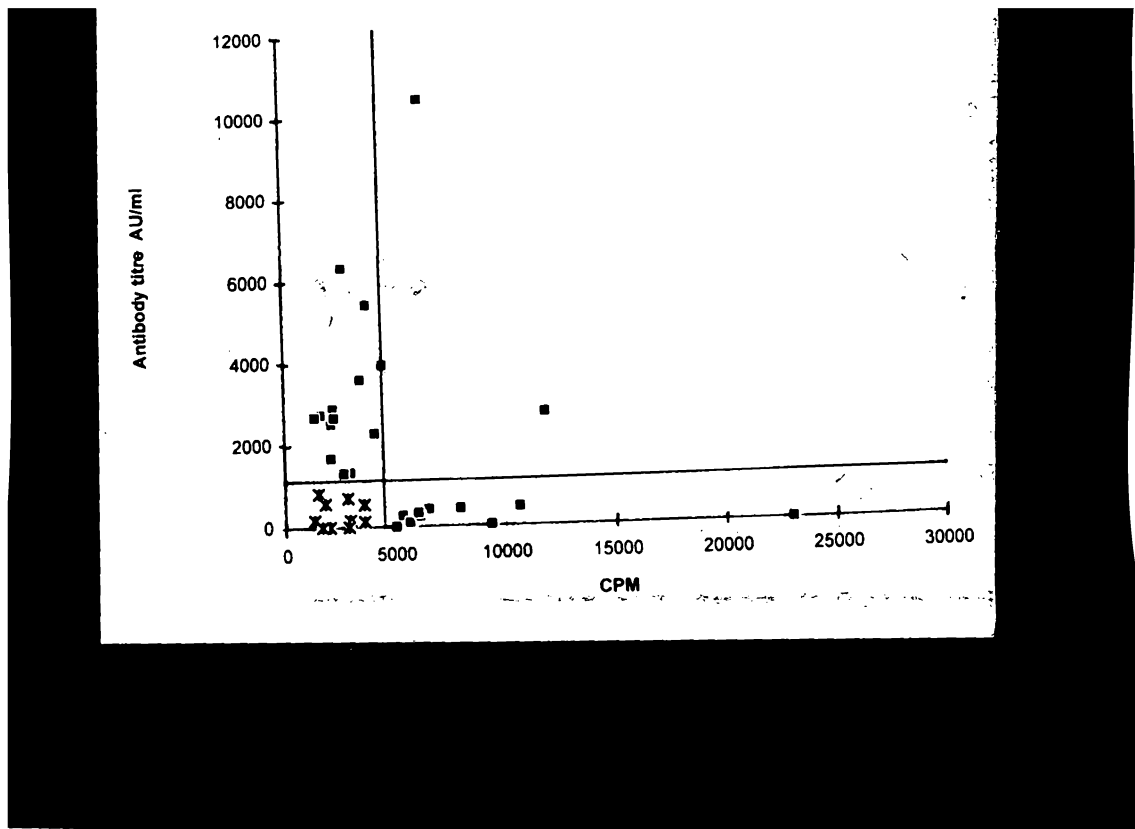


Fig 7. Comparison of the *in-vitro* antigen specific proliferative responses of PBMC and serum anti-65-kDa antibody titers of normal healthy adults and patients of pulmonary tuberculosis . X-axis and Y-axis - Same as in Fig 4.

homogenous Th responses with a predominant Th1 component. The second group of 13 patients revealed a poor antigen specific proliferative response of PBMC, ranging from 1000-4500 CPM, i.e. in the same range as those of the healthy individuals. Their antibody titers varied from 1200-6300 AU/ml, i.e. approximately 17 times the median titre of healthy individuals. Thus these 13 patients formed a second sub group of homogenous Th responses with a predominance of Th2 component. Two of these 13 patients revealed serological evidence of antibodies to HIV, though no other features associated with HIV infection were observed. The third group consisted of only two cases. They revealed excellent antigen specific proliferation of PBMC as well as high antibody titers (figs 2 & 3). Both these patients had antibodies against HIV; Even these patients did not have any other features associated with HIV infection. A very high antibody titre is dependent upon IL-4, a product of Th2 cells. Thus HIV infection appeared to favour a Th2 response to tubercle bacilli.

Analysis of relationship between the *in-vitro* antigen specific proliferative responses of PBMC and response to tuberculin test:

The tuberculin response and the *in-vitro* antigen specific proliferation of PBMC, both of which are considered to be an index of the Th1 type immunity, failed to show any correlation. Half the healthy individuals showed a tuberculin response greater than 10 mm in diameter. Also, a sizeable number of patients, who despite poor proliferative response, showed a strong tuberculin test (fig 8).

Comparison of the responses to therapy:

All the patients, except two cases of pulmonary tuberculosis with Th2 type response, recovered within six months of multidrug treatment. The ESR declines were similar in all the patients irrespective of their Th response status.

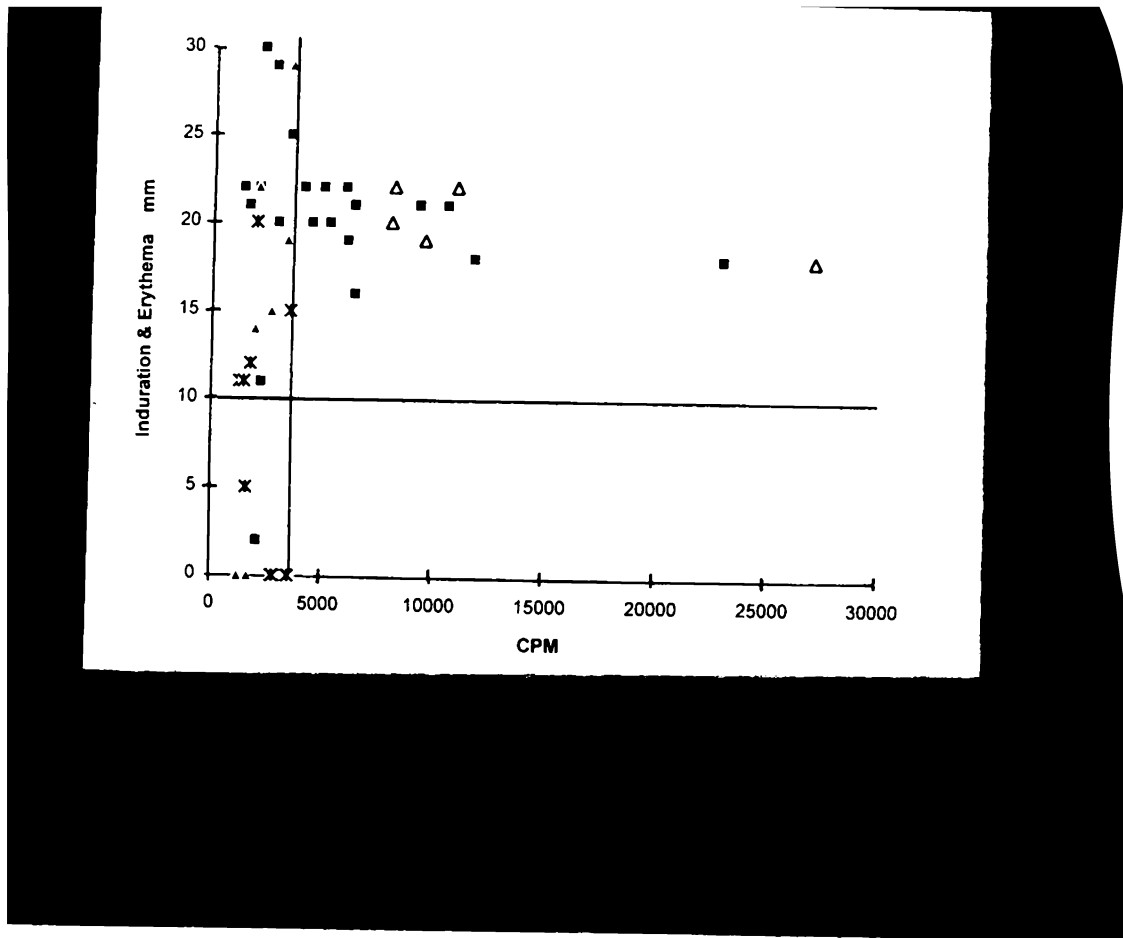


Fig 8. Comparison of response to tuberculin test and the *in-vitro* antigen specific proliferative response of the PBMC of normal healthy adults and patients of tuberculosis.

The symbols of the scatter gram represent the same patients as in Fig 4. The horizontal line in the scatter gram showing outer limit of normal tuberculin response is drawn at 10 mm because that is generally considered as the response of normal healthy individuals. The vertical line is at the same position as in Fig 4.

DISCUSSION

Laboratory diagnosis of tuberculosis, based on the host responses to the bacterial antigen, face two problems which are well illustrated in the present study.

Firstly, in areas of high endemicity, a significant fraction of normal healthy individuals are likely to be inadvertent contacts and as a consequence would reveal significant responses to the mycobacterial antigens. In the present study, practically half the investigated healthy individuals revealed significant responses in all the three tests. Therefore, for diagnostic utility, it is essential that the responses of the patients are significantly greater than those of the healthy individuals.

The second problem is related to the spectral nature of the Th responses in tuberculosis which was described almost twenty years ago by Lenzini *et al* (6). Several investigators feel that the spectrum of immunopathological manifestations of tuberculosis, similar to that of leprosy is not convincing (63). Since the spectral manifestations are believed to be dependent upon polar differentiation of CD4⁺ Th cells into Th subsets, several investigators have studied this aspect. Barnes and Modlin (64) in a

recent review on this issue, claim that the results of different studies are not uniform. Some studies, based on cytokine spectrum of *M. tuberculosis* reactive CD4+ T cell clones prepared from the PBMC of patients of tuberculosis claim polar differentiation (10,11). In contrast, others have reported a wide spectrum and heterogeneity of cytokine secretion by such T cell clones, questioning the polar differentiation (12,13). In still other studies of *in-vitro* antigen induced spectrum of cytokine mRNA expression by PBMC of patients and healthy tuberculin reactors, conflicting results have been reported (14). In none of the studies reported here, a clear cut sharp evidence in the favour of characteristic Th1 or Th2 response in a defined clinicopathological situation is evident.

In contrast to these published reports, in 37/39 patients investigated in this study, a clear cut sharp reciprocal relationship between the *in-vitro* antigen specific proliferative response of PBMC and the anti 65-k-Da titre was evident. In two patients, both responses were elevated, an aberration which could be due to HIV infection (65). The question is whether such data support the conclusion of polar differentiation of

Th subsets in tuberculosis. Complete polar differentiation of Th subsets may not be a common feature in tuberculosis as claimed by several investigators (12-14). Instead, the Th responses could be a mixture of Th0, Th1 or Th2 effecters with a predominance of either Th1 or Th2 components, depending upon the circumstances. In such a situation non-quantitative assays, such as analysis of frequency of *in-vitro* developed Th1 or Th2 clones, amongst those obtained from patients, or *ex-vivo* analysis of cytokine mRNA expression by PBMC of the patients, may not reveal a true picture of the *in-vivo* status. The *in-vitro* antigen induced proliferation of PBMC is an assay of antigen specific IL-2 producing CD4+ T cells, which could be Th0 or Th1 or a mixture of both type effecters. Similarly the anti 65-k-Da titers would be dependent upon levels of IL-4 producing cells which could be Th0 or Th2 or both type effecters. Even though based on reciprocal relationship of the above two responses one cannot conclude polar differentiation, the data still suggests higher frequency of IL-2 or IL-4 producing cells and therefore the two types of responses encountered may indicate a greater Th1 or Th2 component in mixed Th subset responses. In

view of the clear cut sharp reciprocal relationship between the two assays, it is indeed surprising that these assays, have not received as much attention, in the hands of different investigators of immunology of human tuberculosis. Though not necessarily indicative of specific defined subsets, these assays have the advantage of immediate *ex-vivo* analysis and their quantitative nature, over the assays of analysis of cytokine expression by clones of CD4+ T cells and the assays of cytokine mRNA expression by PBMC.

We have studied three types of clinical tuberculosis. The responses of patients of lymph node tuberculosis and tuberculous meningitis were homogenous. Even though responses of patients of pulmonary tuberculosis appeared heterogenous, close scrutiny enabled us to divide 23/25 patients studied into two homogenous groups. Half the patients of pulmonary tuberculosis and all the patients of tuberculous lymphadenitis, whose responses were rich in the Th1 effectors, failed to show antibody titers greater than normal individuals, even though their *in-vitro* antigen specific proliferative responses were very strong. In contrast, the remaining half of the

cases of pulmonary tuberculosis and all the patients of tuberculous meningitis, whose responses were rich in Th2 type effecters, showed high titers of serum anti 65-k-Da antibody. However, their *in-vitro* antigen specific proliferative responses were in the same range as that of

the healthy individuals. Such relatively polar responses were seen uniformly in 37/39 patients investigated.

Tuberculin test, which has been used extensively for diagnosis, appeared to be of little value. Even though median responses of the various groups of patients were higher than those of the healthy individuals, wide variation caused difficulty in correlation. Contrary to expectation, there was no difference in the median tuberculin responses of patients with Th1 or Th2 predominant immunity. The tuberculin response and the anti 65-k-Da antibody titers did not reveal a reciprocal relationship, even though others have reported such a relationship with antibody to other peptide antigens of the organism (66). Similarly, tuberculin response and *in-vitro* antigen specific proliferation of PBMC, both of which are considered to be due to Th1 type immunity failed to show

correlation. Therefore, it was concluded that the tuberculin test was unreliable as a diagnostic test.

It may be noted that if either the *in-vitro* antigen specific proliferation assay or the serum anti 65-k-Da antibody assay was employed singly, diagnosis of tuberculosis would have been missed, despite clinical, radiological and/or bacteriological evidence. However, when both the tests were taken into consideration, 100% of the 39 patients revealed either a higher antibody titre or a stronger *in-vitro* antigen specific proliferative response (except in two cases whose both responses were greater). Thus if one is acquainted with the nature of T subset response in a given clinical syndrome due to tuberculosis, there would be greater confidence in the laboratory diagnosis with two test parameters of Th responses, instead of serological titers alone.

Limitations of data:

The response patterns in three different disease processes have been described here. Even for the three forms of tuberculosis reported in this study, it is necessary to examine a much larger

sample. The controls employed were healthy individuals only; it would be appropriate to examine the *M. tuberculosis* specific responses of individuals suffering from infections other than tuberculosis, in order to ensure specificity.

The antigen employed in the *in-vitro* proliferation assay was the whole sonicate of *M. tuberculosis* containing multiple peptides each having a cross reactive spectrum. Similarly the 65-k-Da antigen employed in the antibody assays is the well known heat shock protein with substantial cross reactivity. Therefore, one may question the specificity of the tests based on such antigenic material. The clear cut reciprocal relationship between the two responses and the characteristic association between a given type of disease and preponderance of either Th1 or Th2 component, that was evident, seem to indicate that the immune regulation in tuberculosis is directed to the specific as well as the cross reactive epitopes. If the precise type of Th subset response exhibited by patients suffering from different clinical disorders due to tuberculosis is identified, diagnosis based on them will be more reliable than the diagnosis based on serology alone. Further, it is

unlikely that infections with organisms having cross reactive epitopes will exhibit the same type of Th subset regulation as seen in a given clinical type of tuberculosis.

Minimal localized pulmonary tuberculosis poses a special problem. All the patients included in our study had localized small lesions. Based on the size, these would have been placed in the reactive groups (RR or RI) of Lenzini classification (6) and one would have expected a Th1 predominant response in all of them. As against this, almost half of our patients had a Th2 predominant response. Even if the HIV infected patients were to be excluded, 40% of the patients had a Th2 response. Lenzini *et al* (6) and Bosen *et al* (67) who have done similar studies on patients with small localized early lung lesions, failed to detect the heterogeneity of the Th responses. The fact that a sizeable fraction of patients with minimal early pulmonary tuberculosis have a Th2 predominant response could be clinically relevant. Following chain of events are believed to take place in the pathogenesis of local and extra pulmonary disease (68). During the interval between the initial implantation of the organism within the alveolus and the

development of cell mediated immunity, there is a tuberculous bacillaemia, resulting in seeding of other parts of lung, lymph nodes and other organs. Once the cell-mediated response is mature, infection at different sites is contained. However, bacillary killing could be incomplete. An important factor in the continued containment of the organisms, is the persistent and intact cell-mediated immunity. Therefore it is likely that the extra pulmonary and the extensive pulmonary disease, both of which are associated with a Th2 predominant response, must have started as cases with localized or minimal disease with a Th2 response predominating in the initial phase itself. If all the early lesions were to be associated with a Th1 type immunity, it would be difficult to explain why extensive caseating local or extra pulmonary disease occurs. Also, it would be impossible to identify from amongst such patients those that are at risk of local or distal spread of the disease. The argument highlights the importance of investigating as prognostic indicators, the two parameters of Th responses, namely *in-vitro* antigen specific proliferation of PBMC and serum antibody titers against a mycobacterial peptide antigen.

The reasons for the observed relatively polar responses of Th subsets in tuberculosis have not been investigated in this study. In lymph nodes the predominant antigen presenting cell is the dendritic cell, which because of its ability to secrete IL-12, is known to favour a Th1 type response (69). Mucosal Th responses are known to be in favour of Th2 type (70), which could be the reason why a significant fraction of cases of pulmonary tuberculosis had a Th2 type response. HIV infection is also reported to favour a Th2 type response (65). Four of the twenty five cases of pulmonary tuberculosis had serological evidence of HIV infection. Two of them revealed a Th2 predominant response and in the other two the anti 65-k-Da titers were very high despite a strong *in-vitro* antigen specific proliferative response of PBMC.

Thus the study suggests the importance of twin tests of Th responses against the tubercle bacilli, namely antigen specific proliferation of PBMC and serum antibody titers against a peptide antigen of the organism in the clinical diagnosis of tuberculosis.

Neither of these parameters finds a place in the current standard protocols of laboratory investigation of tuberculosis.

SUMMARY AND CONCLUSIONS

Parameters of T-helper (Th) responses against *Mycobacterium tuberculosis* were investigated in normal healthy individuals and patients of pulmonary tuberculosis, and tuberculous lymphadenitis and meningitis. The parameters of Th responses tested were, *in-vitro* proliferation of peripheral blood mononuclear cells (PBMC) induced by the sonicate of *M. tuberculosis* H37Rv, serum antibody titers against the 65-k-Da antigen of the organism and the tuberculin test.

Practically half the normal healthy individuals revealed significant responses in the three tests. Therefore the responses of the patients were considered significant only when they were greater than those of normal healthy individuals.

Serum anti 65-k-Da titre and the *in-vitro* proliferation of PBMC revealed a reciprocal relationship in 37/39 patients studied. In two patients both the responses were greater than those of healthy individuals. Thus when both the above tests were taken into consideration, 100% of the 39 patients revealed either a raised antibody titre or a strong *in-vitro* antigen specific proliferative response or both. In contrast, the tuberculin test, which has been

extensively used, appeared to be of little value. Even though the median responses of the patients were stronger than those of the healthy individuals, there was a wide variation. Significantly, no correlation was observed between the tuberculin test and the *in-vitro* antigen specific proliferation of PBMC, even though both the tests are considered to be indicators of Th1 type immunity.

Ten out of 25 patients of pulmonary tuberculosis and all six patients of tuberculous lymphadenitis revealed strong *in-vitro* proliferation of PBMC and low anti 65-kDa titers. Thirteen other patients of pulmonary tuberculosis and all seven patients of tuberculous meningitis revealed high antibody titers and poor *in-vitro* antigen specific proliferation of PBMC. Two patients of pulmonary tuberculosis from this group revealed antibodies against HIV. In the remaining two patients of pulmonary tuberculosis, both the above responses were very strong and they also had anti-HIV antibodies. Tuberculin test results of these three groups of patients showed a wide variation with comparable medians. The results of the single case of miliary tuberculosis were similar to those of tuberculous meningitis.

Thus, the two parameters of Th responses, namely *in-vitro* antigen specific proliferation of PBMC and anti 65-k-Da titre appeared to have diagnostic utility.

APPENDICES

APPENDIX I

Preparation of Media, Reagents and Buffers.

I.1.] Doub and Youmans medium

Asparagine	5.0g
KH ₂ PO ₄	5.0g
K ₂ SO ₄	0.5g
Glycerol	20ml
Distilled water	1000ml

All the ingredients were dissolved in distilled water. After thorough mixing the pH was adjusted 6.8-7.0 by adding appropriate amount of 1N NaOH and Magnesium citrate (1.5g) was added and the solution mixed properly and heated if necessary to dissolve magnesium citrate and then dispensed into tubes or flasks. Medium was sterilised by autoclaving at 15 lbs and 121° C for 20 min.

I.2.] Phosphate buffered saline (PBS) (pH 7.2, 0.15 M)

NaCl	8.0g
K ₂ HPO ₄	1.21g
KH ₂ PO ₄	0.34g
Distilled water	1000 ml

I.3.] Tris-HCl buffer (0.005 M, pH 7.4)

Tris	0.061g
Distilled water	100 ml

Tris was dissolved in 60 ml distilled water, pH was adjusted with 1N HCl to 7.4 and the volume was adjusted upto 100 ml.

I.4.] Carbonate - bicarbonate buffer (0.05 M, pH 9.6)

(Coating buffer)

Na₂CO₃ 0.53g in 100 ml distilled water (0.05 M)

NaHCO₃ 0.42g in 100 ml distilled water (0.05 M)

Approximately 10 ml of Na₂CO₃ (0.05 M) was added to 90 ml of NaHCO₃ (0.05 M) to prepare 0.05 M Carbonate buffer with pH 9.6.

I.5.] Citrate buffer (0.15 M, pH 6.0) (Substrate buffer)

Sodium citrate 0.24g in 100 ml distilled water (0.15 M)

Citric acid 0.21g in 100 ml distilled water (0.15 M)

Approximately 5 ml of citric acid (0.15 M) was added in 45 ml of sodium citrate (0.15 M) to prepare 0.15 M Citrate buffer with pH 6.0.

I.6.] Phosphate buffered saline-T20 (PBS-T20)

(Dilution buffer)

PBS(0.15 M, pH7.2) 1000 ml

Tween-20 0.5 ml

I.7.] Saline-T20 (Washing solution)

NaCl 8.5g

Distilled water 1000 ml

After dissolving NaCl add

Tween-20 0.5 ml

1.8.] O - phenylenediamine dihydrochloride (OPD)

(Substrate solution)

4mg OPD (SIGMA) was dissolved in 10 ml substrate buffer (0.15 M, pH 6.0). 2 μ l of 30% H₂O₂ solution was added and mixed gently. Substrate solution was made fresh each time just before use.

1.9.] Scintillation fluid

1-4 bis (5-phenyl oxazolyl) benzene (POPOP) 0.3016g

2-5 diphenyl oxazole (PPO) 3.968g

Toluene (GR) 1000 ml

PPO and POPOP were dissolved in 800 ml toluene in a volumetric flask thoroughly. After complete dissolution the volume was made upto one litre with toluene.

APPENDIX II

Procedures

II.a.] Estimation of protein by Lowry's method

Reagents:

Reagent A-1

Sodium potassium tartarate 0.2g

Distilled water 10 ml

Reagent A-2

Cupric sulphate 0.1g

Distilled water 10 ml

Reagent A-3

Sodium carbonate 2.0g

Sodium hydroxide (0.1 N) 0.4g

Distilled water 100 ml

Reagent A

1 ml A-1 + 1 ml A-2 was mixed and added to 100 ml of A-3.

Reagent B

Folin-Ciocalteu's reagent (SISCO) was diluted 1:1 with distilled water just before use.

Standard protein solution:

Bovine Serum Albumin (BSA, SIGMA) in the concentration of 200 µg/ml was used as the standard protein solution.

Assay procedure:

A set of clean glass tubes was taken to which 0.1, 0.2, 0.3, 0.4 and 0.5 ml of standard BSA was added in duplicate. The volume of each tube was made upto 5.0 ml with distilled water. Blanks of 0.5 ml distilled water were also set up. The test solutions were also taken in 0.5 ml quantity with appropriate dilutions for the protein estimations. 2.5 ml reagent A was added to each tube. The contents of each tube were mixed and the tubes were allowed to stand at room temperature for 15 min. 0.25 ml of reagent B was added to each tube with vigorous shaking and the tubes were allowed to stand at room temperature for 30 min. Optical density (OD) readings were taken at 660 nm, adjusted against the distilled water blank. A graph of OD against protein concentration was plotted from which the protein concentration of test sample was calculated.

II.b.] Siliconisation of glassware

The glassware to be siliconised was washed thoroughly and dried. The glassware was rinsed with Sigmacote (Silicon solution, SIGMA) by dipping or filling the articles to be coated. They were then drained and air dried. All the glassware for cell culture work was siliconised.

II.c.] Silver staining of protein bands

Reagents

i) Fixative solution

Methanol	50 ml
Acetic acid, 37%	12 ml
Formaldehyde	50 μ l
Distilled water	38 ml

ii) Pre-treatment

Sodium thiosulphate	0.02g
Distilled water	100 ml

iii) Staining solution

Silver nitrate	0.2g
Formaldehyde, 37%	75 μ l
Distilled water	100 ml

iv) Developer

Sodium carbonate	7.5 g
Formaldehyde, 37%	75 μ l
Sodium thiosulphate	0.5 mg
Distilled water	100 ml

v) Destainer solution

Methanol	5 ml
Acetic acid, glacial	12 ml
Distilled water	100 ml

Procedure

- Fixation : The gels were fixed in the fixing solution overnight.
- Washing : Gels were washed in 50% ethanol in distilled water for 20 min. This was repeated thrice.
- Pre-treatment : Gels were pre-treated with sodium thiosulphate for exactly 1 min.
- Washing: Gels were rinsed in distilled water for 20 sec. This was repeated thrice.
- Silver impregnation: Gels were treated with silver nitrate solution for 20 min on a shaker.
- Developing: Gels were developed in developer solution for 5 min, until the bands were developed.
- Washing: Gels were then washed thoroughly in distilled water for 20 min. This was repeated twice.
- Termination of development: Staining was stopped by soaking the gels in the destainer solution.

II.d.] Preparation of IgG fraction from serum

The IgG fraction was separated from the whole serum by ammonium sulphate $[(\text{NH}_4)_2 \text{SO}_4]$ precipitation method. One volume of saturated $[(\text{NH}_4)_2 \text{SO}_4]$ solution was added slowly to two volumes of serum, with constant stirring at 4°C . After 30 min, the solution was centrifuged at 10,000 g for 30 min. The supernatant was discarded, and the precipitate was suspended in the buffer of choice. The solution was dialysed against the same buffer

extensively to remove SO_4^{2-} ions. The absence of SO_4^{2-} ions was tested by BaCl_2 precipitation test. The protein content of the IgG fraction was estimated both by spectrophotometry (extinction coefficient for 1 mg/ml solution at 280 nm taken as 1:4) and by Lowry's method.

II.e.] Standardisation of conjugate dilution

The dilution of conjugate to be employed in a given ELISA was standardised as follows:

- Solution of required antigen (eg. human IgG for anti human HRP conjugate) was prepared in coating buffer (Appendix I.4.) at concentrations 1, 5 and 10 $\mu\text{g/ml}$.
- 10 mg/ml BSA solution in coating buffer was prepared.
- 100 μl of each concentration of antigen was added to the wells of the ELISA plate (Maxisorp, NUNC) in adequate numbers to titrate various dilutions of the conjugate. 100 μl of the BSA solution was added to another set of wells also in sufficient numbers as controls for the various dilutions of the conjugate.
- Plate was covered and incubated in a moist storage box overnight at 4°C.
- Next day, the well contents were discarded.
- All the wells were then filled with 2% gelatine in coating buffer, the blocking solution and incubated at 37°C for 1.5h.

The wells were washed 5-6 times with saline-T20.

- Three suitable dilutions (such as 1:15,000, 1:40,000 and 1:60,000) of the conjugate to be tested, were made in dilution buffer (Appendix I.6.)
- 100 μ l of each conjugate dilution was added to the wells. PBS-T20 was added to a set of wells as conjugate blank and incubated at 37°C for 1h.
- The wells were then washed 5-6 times and 100 μ l of the substrate solution (Appendix I.8.) was added. The plate was kept in dark at room temperature for 15 min.
- When optimal colour developed, the reaction was stopped with 50 μ l of 2.5 N HCl and absorbance was read at 492 nm in an ELISA plate reader.
- A₄₉₂ values were plotted against the dilution of conjugate.

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SYNOPSIS

A SYNOPSIS OF THE THESIS TO BE SUBMITTED TO
THE UNIVERSITY OF MUMBAI
FOR THE DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY

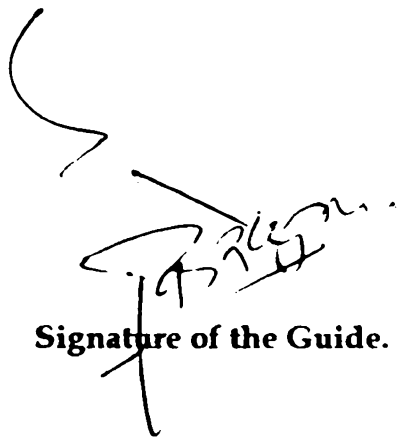
Title of the Thesis : Study of helper T cell parameters in clinical tuberculosis.

Name of the candidate : Ms. Manjusha D. Mankar.

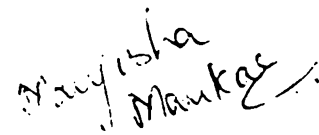
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Signature of the Guide.



Signature of the candidate

INTRODUCTION

Tuberculosis is an ancient disease, known to mankind at least for the last 6000 years. It has been for many centuries the most important of human diseases in its global prevalence, dreadful morbidity and massive mortality.

Tuberculosis, which is essentially a chronic mycobacterial disease, develops through an immunological spectrum that ranges from a state of high resistance to an opposite extreme of low resistance. Lenzini (1) has established a spectrum of progressive human tuberculosis on clinical and immunological grounds. Patients fall into one of the four groups consisting of a polar reactive group (RR) associated with a well developed cell mediated immunity (CMI) and opposite unreactive group (UU) in which CMI is absent, and two intermediate groups, one towards the reactive end (RI) and the other towards unreactive end (UI). The number of acid fast bacilli in the infected tissues increases towards the unreactive pole as CMI diminishes and this is accompanied by an increase in antibody levels. The RR form is characterised by lymphocytes and epitheloid cells in the lesions. In the UU form, bacteria and lesions spread rapidly in lungs and other organs, thus indicating the importance of CMI in progressive mycobacterial disease such as tuberculosis.

Central event in the generation of both humoral and cellular immune responses is activation and clonal expansion of CD4⁺ Th cells. A major sub-division of CD4⁺ Th cells based on differences in their pattern of cytokine production has been proposed (2). Th1 cells which secrete IL-2, IFN- γ and TNF- β ; are the main cells particularly in CMI and Th2 cells which make predominantly IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 are the main cells responsible for T help to antibody production. T cells expressing cytokines of both patterns have been called as Th0 cells (3).

Analysis of behaviour of CD4⁺ T cell clones have indicated that Th1 and Th2 cells may differ in their requirements for costimulatory signal(s) and thus for antigen presenting

cells (APCs) (4). It might be suggested that the type of APC involved in priming the CD4⁺ T cell may also influence the class of effector response observed. B cells, macrophages and dendritic cells differ in their ability to take up and process antigen, may differ in costimulatory signals and differ in MHC class II density. They would also differ in terms of cytokines and other products. All these factors might contribute to selective activation of TH1- or Th2 - like cells *in vivo* when different cells present antigen. It can be therefore suggested that in case of the disease like tuberculosis, which occurs in different organs or multiple organs in a single patient, the outcome of the immune functions would be influenced greatly by organ specific antigen presenting cells.

Therefore, a possibility exists that immune responses may differ at the local and systemic sites in patients with tuberculosis. In view of these, following aims and objectives have been set-

AIMS AND OBJECTIVES

- To study delayed type hypersensitivity response to PPD in patients suffering from tuberculosis of different organs.
- To evaluate lymphoproliferative responses of peripheral blood lymphocytes against the sonicate of *M.tuberculosis* in patients suffering from tuberculosis of different organs.
- To assess serum antibody responses against the immunodominant 65-kDa antigen of *M.tuberculosis* in patients suffering from tuberculosis of different organs.

MATERIALS AND METHODS

1. **Patients :** Venous blood was obtained from patients of newly diagnosed pulmonary tuberculosis (n=25), tuberculous meningitis [TBM] (n=7), lymph node tuberculosis (n=6) and miliary tuberculosis (n=1). All the cases were either bacteriologically (n=18), radiologically (n=15) or histologically (n=6) confirmed.
2. **Microorganisms :** *Mycobacterium tuberculosis* H₃₇Rv (NCTC 7416) was purchased from National Collection of Type Cultures, Collindale, London. The strain was maintained on Lowenstein Jensen's medium slants and cultivated on the Doub and Youman's medium (5).
3. **Animals :** Adult Albino rabbits weighing 1.5 - 2.0 kg were purchased from Haffkine Biopharmaceutical Corporation Limited, Mumbai and used for raising antisera against 65-kDa antigen of *M.tuberculosis*.
4. **Preparation of antigen (whole sonicate of *M.tuberculosis* H₃₇Rv) :** Five week old culture of *M.tuberculosis* in the Doub and Youman's medium was harvested and then subjected to 2.5 Mrad gamma irradiation in a Co⁶⁰ chamber. The irradiated cells were washed with phosphate buffered saline (PBS) and subjected to sonic disintegration in a sonicator (Branson Sonifier, B-30, 20kHz, 50% duty cycle) for 45 min. and spun at 50,000 g for 1h. The supernatant was filtered through 0.22µm membrane filter (Millipore, USA) and its protein content was estimated by the method of Lowry *et al* (6). It was then aliquoted and stored at -20°C and used as "test antigen".
Whenever required the sonicate was subjected to lysozyme-papain digestion as described earlier (7).
5. **Preparation of rabbit anti-65kDa antibody :** The 65-kDa antigen in the sonicate of *M.tuberculosis* H₃₇Rv was purified employing the method described earlier (7) and emulsified in Freund's incomplete adjuvant. A 2.5 kg rabbit was immunised repeatedly at 10 day intervals with 1.0ml. emulsion. At each time, till a precipitin titre of 1:16 was reached.

6. Delayed type hypersensitivity (DTH) test : 0.1ml. of 10 TU tuberculin (PPD) was injected intradermally in the forearm of the patients and redness and induration were measured after 48h.
7. Lymphoproliferation assay : Lymphoid cells from the peripheral blood were obtained by Ficoll Hypaque density gradient centrifugation. Lymphoproliferation assay was carried out employing standard microculture technique (8).
8. Assay for anti 65-kDa antibody levels : Antibody levels against 65-kDa antigen of *M.tuberculosis* were assayed by sandwich ELISA technique.

ELISA plate (Maxisorp- Nunc, Denmark) wells were coated with rabbit antibody against 65-kDa antigen of *M.tuberculosis* by incubating with 100µl/well of the antibody solution (IgG concentration 500µg/ml) overnight at 4°C. Next day the well contents were discarded and the well surfaces were blocked with 2% gelatine for 1 ½ h at 37°C. Then they were washed 5-6 times and 100µl of lysozyme-papain digested *M.tuberculosis* sonicate (1mg/ml) was added to the wells and incubated for 2h at 37°C. The wells were again washed and 100µl of different dilutions of test serum along with appropriate controls (positive control serum and PBS -Tween 20 as negative control) were added and incubated for 1 ½ h at 37°C. These were washed and 100µl of anti-human HRP conjugate (dilution 1:40,000) was added to the wells and incubated for 1 ½ h at 37°C. The plate was then washed and 100µl of substrate solution (OPD - 4mg/10ml, H₂O₂ - 2µl/10ml) was added to the wells and kept at RT in the dark for 12-15 min. The reaction was stopped with 2.5M HCl and read at 495 nm. Antibody levels were expressed as absorbance units (AU) per ml.

RESULTS

Cellular and humoral responses in clinically diagnosed cases of tuberculosis were studied.

The DTH response was seen in the patients by injecting PPD intradermally and measuring redness and induration after 48h. Venous blood was obtained from the patients and lymphoproliferation responses against the sonicate of *M.tuberculosis* H₃₇Rv carried out. Also, the serum samples were assayed for antibodies against the immunodominant antigen in the sonicate, the 65-kDa antigen, by ELISA.

Along with the patients normal healthy individuals were also studied as controls.

I. Responses of asymptomatic normal healthy controls:

The median values and the ranges of the three responses of asymptomatic controls were as follows-

T HELPER CELL RESPONSES OF ASYMPTOMATIC HEALTHY CONTROLS

Response	Median	Range
DTH response (mm)	8	0 - 20
<u>Lymphoproliferation response</u>		
CPM	2320	1069 - 4291
Stimulation Index (SI)	2.35	1.28 - 3.5
Anti 65-kDa antibody response (AU)	177	12 - 827

II. Responses of patients :

A) Pulmonary tuberculosis: A considerable variation in the responses was seen. The median values and ranges of 25 cases are given below. However on careful analyses, the responses could be separated into three distinct patterns.

T HELPER CELL RESPONSES OF PATIENTS WITH PULMONARY TUBERCULOSIS

Response	Median	Range
DTH response (mm)	21	2 - 30
<u>Lymphoproliferation response</u>		
CPM	3778	1145 - 9214
Stimulation index (SI)	4.4	1.3 - 23.0
Anti 65-kDa antibody response (AU)	1679	10 - 10,427

i) Patients having responses suggestive of Th1 type :

The median antigen specific T cell proliferative responses were higher (median stimulation index approximately 2.5 fold) than the asymptomatic individuals. In contrast, the anti-65-kDa antibody responses were of the same order or lower than those of the asymptomatic individuals. The median DTH response of this population was greater than that of the asymptomatic healthy individuals. The median and the ranges are shown below.

10/25 cases showed this type of response.

T HELPER CELL RESPONSES OF PATIENTS SHOWING TH1 TYPE RESPONSE

Response	Median	Range
DTH response (mm)	20	16 - 22
<u>Lymphoproliferation response</u>		
CPM	5355	3458 - 9214
Stimulation index (SI)	6.25	5 - 23
Anti 65-kDa antibody response (AU)	187	10 - 432

ii) Patients having responses suggestive of Th2 type :

The antibody responses of these patients were higher (median antibody titre approximately 15 fold) than those of the controls, while the T-cell proliferative

responses were of the same order as those of asymptomatic cases. Again the DTH responses of these patients were greater than those of the asymptomatic healthy individuals. However they did not differ significantly from the DTH responses of the previous group. The median and the ranges are shown below.

13/25 cases showed this type of response.

T HELPER CELL RESPONSES OF PATIENTS SHOWING TH2 TYPE RESPONSE

Response	Median	Range
DTH response (mm)	22	2 - 30
<u>Lymphoproliferation response</u>		
CPM	2185	1145 - 4308
Stimulation index (SI)	2.6	1.3 - 4.4
Anti 65-kDa antibody response (AU)	2676	1311 - 6316

iii) Patients with mixed type of T-helper responses :

Both the antibody as well as the T- cell proliferative responses of these patients were higher than those of the asymptomatic individuals. Again, the DTH responses of these patients were greater than those of the asymptomatic individuals but did not differ significantly from those of the previous two groups. The median and the ranges are shown below.

2/25 cases showed this type of response.

T HELPER CELL RESPONSES OF PATIENTS SHOWING MIXED TYPE RESPONSE

Response	Median	Range
DTH response (mm)	19	18 - 21
<u>Lymphoproliferation response</u>		
CPM	6308	5135 - 7481
Stimulation index (SI)	9.10	6.4 - 11.8
Anti 65-kDa antibody response (AU)	6579	2732 - 10,427

It is significant to point out that 2/13 cases with Th2 type of responses and both the cases with mixed T-helper cell responses were seropositive for HIV antibodies. Thus, the HIV positive individuals either induced a Th2 type of response or induced the patients to make higher levels of antibodies despite a Th1 response.

B) Lymph node tuberculosis :

All the six cases of isolated lymph node disease showed responses suggestive of Th1 type. The T cell proliferative responses were uniformly higher (median stimulation index 4 fold) and the antibody responses were comparable or even lower than those of the asymptomatic individuals.

The DTH responses of these patients were greater than those of the asymptomatic individuals but did not differ significantly from the responses shown by patients with pulmonary tuberculosis. The median and the ranges are shown below.

T HELPER CELL RESPONSES OF PATIENTS WITH LYMPH NODE TUBERCULOSIS.

Response	Median	Range
DTH response (mm)	20	18 - 22
Lymphoproliferation response		
CPM	6188	5547 - 17,160
Stimulation index (SI)	10.3	8 - 27
Anti 65-kDa antibody response (AU)	36	20 - 294

C) Tuberculous meningitis (TBM):

All the seven cases of TBM showed a Th2 type response, wherein the T cell proliferative responses were lower and the antibody responses were higher (median antibody titre approximately 20 fold) than those of asymptomatic individuals. The DTH responses were not significantly different from those of asymptomatic individuals.

T HELPER CELL RESPONSE OF PATIENTS WITH TBM

Response	Median	Range
DTH response (mm)	15	0 - 22
<u>Lymphoproliferation response</u>		
CPM	1826	1204 - 3215
Stimulation index (SI)	2.0	1.2 - 3.5
Anti 65-kDa antibody response (AU)	3769	2714 - 7927

D)Miliary Tuberculosis :

The single case of miliary tuberculosis studied, showed a distinct Th2 type response; (T cell proliferation : mean CPM 1229 ± 106 , SI 2.3; Anti 65-kDa antibody titre 8073.84 AU).

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