Chapter – 1

Preface
Mycobacterium tuberculosis that causes tuberculosis (TB) is the most dreaded scourge of mankind from the antiquity and is responsible for 8 million new cases and 3 million deaths annually (Srivastava and Srivastava, 2000). As we enter the new millennium, the incidence of TB in the world is expected to increase from 10.2 million cases in the year 2000 to 11.9 million cases in 2005. It is estimated that about 1/3rd of the population is infected and another 300 million will be infected in the next decade. About 95% of TB cases and 98% of TB deaths occur in developing countries (Figure 1).

The evidence of spinal tuberculosis has been encountered in Egyptian mummies. It has been for many centuries, the most important of human infections, in its global prevalence, with devastating morbidity and massive mortality. Rising incidence of TB due to resurgence of AIDS and emergence of multi-drug resistant strains of M. tuberculosis have assumed frightening catastrophic dimension (Styblo, 1991).

Various types of vaccines have been developed against pathogens. When an antibody response is able to confer protection, subunit or inactivated vaccines are efficient. In contrast, in the case of TB and certain other infectious diseases, killed pathogens are not protective (Winter et al, 1991).
Figure 1: TB: The continuous scourge of humankind.

**TUBERCULOSIS**
- A global threat:
- 2 billion infected
- 8 million new cases annually
- 2 million deaths annually
- AIDS & TB, a dangerous liaison:
  - >10 million co-infected
  - >¼ million additional deaths annually

**CONTROL MEASURES**
- Chemotherapy:
  - Works, but poor compliance
  - Increasing incidences of MDR-TB
  - >3% of all TB cases in several countries
  - 50 million infected with MDR-organisms
  - 100-fold increase in cost
- Vaccination:
  - Prevents severe forms of childhood TB
  - Ineffective against adult pulmonary TB

Figure 2: Selected interactions between lymphocytes and mycobacteria-infected macrophages. Surface molecules play a critical role in facilitating cytokine release but are not shown here. Solid lines indicate activities stimulatory to mycobacterial killing. Whereas dashed lines indicate activities inhibitory to mycobacterial killing. Mycobacteria (AFB) infect the macrophage and lead to production of IL-12 to signal T and NK cells to produce interferon-γ. CD4⁺ T cells produce interferon-γ, which activates the macrophage to produce TNF-α, kill intracellular bacteria, and produce more IL-12. CD4⁺ T cells also produce IL-2, which expands the number of T cells and NK cells available NK cells release GM-CSF and TNF-α, which also activate macrophages. IL-10 is produced by macrophages and T cells to down-regulate the effects of interferon-γ and TNF-α. TH2 cells produce IL-4 and down-regulates and T_{H1} cell response. Infected macrophages can produce PGE₂, which decreases killing of mycobacteria but is overcome by interferon-γ. Scanned from *Sem. in Respir. Infect.*, 1996 (11) p. 217.
Figure 3: Cell-mediated immunity enables the host to produce large numbers of highly activated macrophages in the tuberculous lesion. It is more fully developed in mouse resistant to pulmonary tuberculosis than in those susceptible to the infection. The mechanism involves macrophage ingestion of tubercle bacilli and presentation of bacillary antigens to specific T lymphocytes (A), which then secrete lymphokines that attract monocytes and macrophages (B) and activate them to kill the bacilli (C). Such activation can be variable, with some macrophages only partially activated and weakly microbicidal, and other macrophages highly activated and potently microbicidal. Scanned from *Hosp. Pract.* 1993; p.55.
Protective immunity to intracellular bacteria such as mycobacterium and salmonella has long been known to depend on cell-mediated immunity (CMI). The major effector mechanism of CMI is thought to be the activation of infected macrophages by type 1 cytokines, particularly interferon-γ (IFN-γ). IFN-γ is produced by natural killer (NK) and Th1 cells and its production is regulated by interleukin-12 (IL-12), which is released by macrophages as well as dendritic cells. IFN-γ, together with monokines such as tumour necrosis factor-α (TNF-α), activates microbicidal mechanisms of macrophages that are responsible for the control and elimination of the intracellular infectious pathogen (Figure 2). In addition, CMI may involve cell-mediated lysis of infected macrophages by cytotoxic T cells and perhaps NK cells (Figure 3). Effective CMI typically leads to the containment of the pathogen inside highly organized granulomatous lesions (Doherty and Andersen, 2000). In other instances, this type of immunity can best be achieved by immunization with a live vaccine. Such live vaccines are believed to better deliver the proteins to antigen presenting cells (APCs), especially when the vaccines are in vivo-replicating intracellular microorganisms. They represent a greater pool of antigens that presumably should cover wider range of T-cell repertoires. They increase the resistance against challenge with virulent M. tuberculosis (Abou-Zeid et al, 1988; Andersen et al, 1991; Andersen, 1994). They are generally more cost-effective to produce. Infectious agents activate in the host a highly organized response that controls pathogen dissemination and minimizes tissue damage. Early phases of the response require the immuno-regulatory influence of γδ T cells (Mosmann et al, 1986; Cherwinski et al, 1987) that contribute to the overall ability of infected hosts to eliminate infecting intracellular microbes, i.e., Listeria, Leishmania or Mycobacterium (Coffmann, 1986; Coffmann and Carty, 1986; Cher and Mosmann, 1987; Fong and Mosmann, 1989). This effect involves the limitation of lesion size, possibly via, control of polymorphonuclear leucocytes housing and functions at the infection site (Gajewski and Fitch, 1988; Fong and Mosmann, 1989).

The cross talk between T cells and macrophages also contributes to the outcome of the disease (Kelso and Gouch, 1988). Possibly influencing individual resistance to infections. Activated murine CD4+ T cells comprise at least two functionally distinct subsets of cells (Gajewski and Fitch, 1988). Th1 cells that secrete, IL-2 and IFN-γ upon
activation but not IL-4 or IL-5, and Th2 cells that produce IL-4 and IL-5 but not IL-2 or IFN-γ. The differential cytokine secretion profile of these CD4⁺ T cells correlates with different effector functions exerted by these cells: Th1 cells mediate delayed type hypersensitivity (DTH) responses (Coffmann, 1986), and Th2 cells provide superior help for antibody production by B-cells (Mosmann et al., 1986). There is some support for the notion that Th1 and Th2 cells are progeny of Th0 cells, which can produce IL-2, IFN-γ, IL-4 and IL-5 simultaneously (Cherwinski et al., 1987) (Figure 4).

Protection against infection with *M. tuberculosis* H37Rv can be considered in two stages. an early innate resistance followed by an acquired protective response. In both stages, the macrophage is the primary effector cell with the capacity to restrict growth of the intracellular mycobacteria. In inbred mice strains, the non-immune innate response to BCG appears to be controlled by a single gene, Bcg/Ity/Lsh, which confers macrophage resistance (Vidal et al., 1993). It has long been established that the development of acquired immunity to tuberculosis is mediated through a specific T cell-mediated immune response (CMI) in which the co-operative action of antigen specific T cells and macrophages ultimately controls infection by inhibiting growth of the phagocytosed mycobacteria (Orme et al., 1993).

The modulation of macrophage activity by T cells is mediated by secreted lymphokines, which are required for induction of potent antimicrobial activity. This interaction between macrophage and lymphocyte involves the presentation of processed mycobacterial antigens to antigen-specific T cells. Appropriately sensitized CD4⁺ T cells secrete cytokines, which activate macrophages to express enhanced anti-mycobacterial activity. CD8⁺ cytolytic T cells are also thought to play an important role in protective immunity by releasing organisms, which are then taken up by recently activated macrophages (Carpenter et al., 1995). Following an initial response to antigen, some lymphocytes acquire altered properties and persist for prolonged periods. The collective behaviour of these cells, termed immunologic memory constitutes one of the cardinal features of the vertebrate adaptive immune system. Although memory T cells are known to exist, the properties that distinguish them from their native precursors are only known to a limited extent. If infection is initiated when memory cells are already present (as a result, say, of previous infection with the same organism or vaccination), these cells are capable...
Figure (4). Schematic representation of cytokines influencing the development of antigen activated naive CD4+ T cells into Th1 and Th2 cells.

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of immediately mounting a cytolytic attack on infected cells and secreting IFN-γ and IL-2. Naïve cells, in contrast, have to undergo proliferation and differentiation into effector cells, a process that often takes many days during which infection progresses. And even though memory cells appear to have a greater propensity to undergo antigen-driven cell death, the surviving cells can proliferate and probably maintain the memory population. These properties suggest that current efforts to develop vaccines for eliciting CD8+ T cells responses should focus on immunization strategies and immunogens that enhance the generation and survival of memory T cells (Cho et al, 1999).

Clinicians have two tools in hand to fight with this malady namely the chemotherapy and vaccinotherapy. The increasing frequency of drug-resistant strains has fuelled deadly outbreaks of disease that are poorly responsive to chemotherapy. Thus it has limited the use and scope of chemotherapy alone in the control of tuberculosis. Short-course chemotherapy for TB is one of the most effective measures available in developing countries and should be applied widely and efficiently as far as possible. But the main antituberculosis drugs can also cause adverse reactions such as, arthralgia during pyrazinamide administration. Thrombocytopenic purpura, shock, haemolytic anaemia or acute renal failure can occur due to rifampicin treatment. Similarly ethambutol can cause retrobulbar neuritis (Bulletin of the International Union Against Tuberculosis and Lungs Disease, 1988). Thus, there appears to be limited scope for eradication or control of tuberculosis through drugs alone. There is a need for application of new principles in the fight against TB. It is well established that a vaccine, if it works, is the most effective tool to control an infectious disease (ECC/STD Initiative Report of the Expert Panel IX, 1996). For a new vaccine to have any credence as a potential replacement for BCG, it is imperative to demonstrate (i) it can prevent this caseating disease and instead induce a cellular response in the lungs similar to that induced by BCG, and (ii) it can protect the animal over the long term, at least to the extent provided by BCG.

Soon after the discovery of tubercle bacillus by Robert Koch, the search for a suitable immunizing agent against tuberculosis started. Three distinct types of vaccines were initially used from time to time.
1) Preparations containing small number of live *M. tuberculosis* bacilli (Baldwin *et al*, 1998) never met with success proved to be hazardous, since only few live virulent bacilli were capable of producing the overt disease hence, discarded. Preparations containing non-pathogenic mycobacteria to men but pathogenic to other species of animals (Baldwin *et al*, 1998) was studied with some success. Hart *et al* (1967) gave an assessment of tuberculosis vaccines used in adolescents in Great Britain and concluded that, although both BCG and vole bacillus vaccines, have so far produced similar degree of protection, but lupus has been observed to develop at the site of vaccination in some of the participants given vole bacillus vaccine, hence such an attempt was never recommended for mass use.

2) Attenuated variants of originally virulent strains of tubercle bacilli were pathogenic to men (Weiss, 1959). Presently existing vaccine BCG (Bacillus Calmette-Guérin) developed by Calmette and Guérin (1921) originated from a virulent bovine strain of the tubercle bacillus that had been isolated by Nocard form of a cow with tuberculous mastitis. This is a strain of *M. bovis* attenuated by 230 serial subcultures in a glycine-bile-potato medium during 1908-1918. It is officially recommended in 182 countries-territories. Despite its recommended use, BCG has not been able to control tuberculosis. There have been several scientifically valid controlled trials of BCG throughout the world. The protection rates in these trials have risen from 0-80% (Suderland, 1971).

3) Heat killed or attenuated whole cell vaccines prepared from *M. tuberculosis* and *M. bovis* can be used to control tuberculosis to some extent.

Live mycobacterial vaccines induce higher levels of antituberculous resistance in experimental animals than either whole dead bacilli or their cell components, even when presented in a suitable adjuvant (Collins, 1984). This difference is speculated to be due to the secretion of some proteins in the surrounding media during growth known as secretory proteins (Andersen *et al*, 1991). These proteins may induce cell mediated immunity responses and antituberculous resistance (Andersen *et al*, 1991). However, on vaccination with live bacilli, these proteins will be continuously synthesized and secreted into the surrounding tissues and may provide consistent sources of antigen in the body. Studies in
animal models have demonstrated that only live multiplying mycobacteria efficiently induce protective immunity (Collins, 1974; Hubbard et al., 1991; Andersen, 1991, 1994).

Since *M. habana* is also a very promising immunogenic agent having several immunodominant proteins common with *M. tuberculosis* and *M. leprae*, it requires thorough investigation to look for safety of *M. habana*, and to find out the role of it in affording protection against these mycobacterial infections, leading to development of a live vaccine for tuberculosis. The existing vaccine against TB namely BCG (Bacillus Calmette and Guérin) has not been able to control TB; because, (i) after BCG vaccination, it is difficult to use delayed type hypersensitivity (DTH) skin tests for diagnostic or epidemiological purposes, (ii) vaccine efficacy is very variable in different parts of the world. It was realized early that BCG might work less in populations with a background of sensitization to environmental mycobacteria and it was for this reason that a controlled clinical trial was mounted in South India, where such sensitization was known to exist. It was nevertheless remarkable that essentially zero protection was found in this population, with the exception of the youngest vaccinees (Tripathy, 1987). Keeping this controversial status in mind, we used *M. habana* (*M. simiae* serovar I), as a vaccine strain for protection in mouse against experimental *M. tuberculosis* challenge. This strain was found to offer consistent protection against *M. leprae* challenge in the mouse foot pad (Youmans and Youmans, 1957; Singh et al., 1981, 1985, 1989; Gupta et al., 1987) affords consistent protection of mice against *M. ulcerans* challenge (Wolinsky, 1984; Chaturvedi et al., 1999). It generates strong cell mediated immune responses and shares several immunologically important proteins with *M. tuberculosis* and *M. leprae* (Singh et al., 1988). This also provided significant protection against *L. donovani* challenge in hamsters.

**Objectives:**

1. To study the protection rendered by *M. habana* in inbred mice

   General appearance, weekly body weights, postmortem lesions in visceral organs, weight of visceral organs, histopathological examination of lungs, liver and spleen sections, viable count of Acid Fast Bacilli (AFB) in lungs, spleen and liver, percent survival time and mean survival time (MST) of the inbred mice were studied.
2. Studies on immune responses with special reference to the cytokine production by CD4\(^+\) and CD8\(^+\) specific T-cells

Chromium release assay to measure the lymphocyte mediated cell killing \textit{in vitro}, cell mediated immune responses with special reference to the cytokine production by CD4\(^+\) and CD8\(^+\) specific T cells and analysis of antibody response of AKR mice immunized with \textit{M. habana} were performed.