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Review of Literature
2.0 General Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis*. It is one of the oldest human afflictions which has plagued mankind throughout recorded and archeological history leading to infectious killer of youth, children and adults and second most common infectious disease worldwide (WHO report 1995), despite the global use of live attenuated vaccine and several antibiotics. It has been estimated that there are nearly 1.7 billion individuals infected with *Mycobacterium tuberculosis* throughout the world, approximately 20 million of these are active cases and three million die each and every year leading to eight million new cases annually and three million of these are infectious (WHO report 2002). In India, out of the 950 million inhabitants, 300 million are infected with *M. tuberculosis*, 12 million have active tuberculosis, three million are infectious and half a million die each year (WHO 2002, and 2004).

One third of world’s population is currently infected (Kochi *et al.*, 1994; WHO report, 2003) with *M. tuberculosis* in which 20 million of these are infected with active cases. However these can further infect 50-100 million people (Largely children) annually.

The mortality due to disease is approximately 3 million annually; at least 80% of those are in the developing countries (Smith Issar, 2003). Thus, tuberculosis is still a major cause of disease and mortality whose elimination will be impossible as long as poverty, overpopulation and malnutrition is concerned (Smith, 2003). TB is now becoming the leading cause of death
among HIV positive people where it kills much more rapidly with a fatality of
80% population.

The epidemic of HIV infection has radically changed the epidemiology of
tuberculosis. Because of its ability to destroy the immune system, HIV has
emerged as the most significant risk factor for progression of dormant TB
infection to clinical disease (Selwyn et al., 1989).

2.1 ORIGIN:

The origin of *Mycobacterium tuberculosis*, the causative agent of TB, has
been subject of much recent investigations and it is thought that the bacteria
in the genus *Mycobacterium*, like other actinomycetes, were initially found in
soil and that some species evolved to live in mammals. The domestication of
cattle, thought to have occurred between 10,000 and 25,000 years ago, would
have allowed the passage of a Mycobacterial pathogen from domesticated
livestock to humans and in this adaptation to a new host. The bacterium
would have evolved to the closely related *M. tuberculosis*. Specifically, it has
been hypothesized that *M. bovis*, which causes a TB-like disease in cattle
was the hypothetically evolutionary precursor of *M. tuberculosis* (Stead,
1997). This hypothesis is now considered doubtful in the light of new data,
since it was formulated before the genome in the *M. tuberculosis* complex
including the human and animal pathogens *M. africanum, M. microti* and *M.
Canetti* as well as *M. tuberculosis* and *M. bovis*, were characterized by DNA
sequencing and related methods. These studies have shown greater than
99.9% similarity of DNA sequencing among the members of the *M.*
tuberculosis complex (Brosch et al., 2002). But the existence of rare synonymous single nucleotide polymorphisms (sSNP) allows discrimination between these closely related bacteria. sSNP analyses suggest that M. bovis evolved at the same time as M. tuberculosis (Sreevatsan et al., 1997) and a study of the distribution of deletion and insertions in the genomes of the M. tuberculosis complex provides strong evidence for the independent evolution of both M. tuberculosis and M. bovis from another precursor species, possibly related to M. canetti (Brosch et al., 2002).

It is thought that the progenitor of the M. tuberculosis complex, comprising M. tuberculosis, M. bovis BCG, M. africanum and M. microti arose from a soil bacterium and that the human bacillus may have been derived from the bovine from following the domestication of cattle. The complex lacks inter strain genetic diversity and nucleotide changes are very rare.

This is important in terms of immunity and vaccine development as most of the proteins will be identical in all strains and therefore antigenic drift will be restricted. On the basis of the systematic sequence analysis of 26 loci in a large number of independent isolates, it was concluded that the genome of M. tuberculosis is either unusually inert or that the organism is relatively young in evolutionary terms.

2.2 Establishment of Mycobacterial infections

Since 1882, Robert Koch identified Mycobacterium tuberculosis as the causative agent of TB, M. tuberculosis can be best described as an obligate
aerobe, generally characterized by a long replication time and a cell wall containing abundant lipids and waxes that provide hydrophobic characters, acid fast properties and intracellular survival (Gebbardt et al., 1996). There are five closely related *Mycobacteria* grouped in the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti* (Van Soolingen et al., 1997; Van Soolingen et al., 1998). Members of the *M. tuberculosis* complex can all cause the disease in humans, although *M. tuberculosis* is the most prevalent. The natural reservoir of *M. tuberculosis* and *M. africanum* is limited to humans and that of *M. microti* is mainly limited to small rodents (Kremer et al., 1998). In contrast, *M. bovis* can cause disease in a wide range of wild and domestic animals as well as in humans (Brosch et al., 2002; Morris et al., 1994).

Transmission of TB in man usually occurs via air borne microscopic droplet nuclei (1-5 μm diameter) containing *M. tuberculosis*. The infectious droplet nuclei are inhaled and lodge in the pulmonary alveoli (Loudon and Roberts, 1967; Riley et al., 1995), where, then the bacilli are phagocytosed by alveolar macrophages and remain in the phagosome of these cells (Armstrong and Hart, 1975). Following phagocytosis, *M. tuberculosis* replicates slowly but continuously, and is spread to the neighboring lung tissue and through lymphatic vessels to draining hilar lymph nodes (Frieden et al., 2003).

It is not fully understood how *M. tuberculosis* can survive and replicate intracellularly in macrophages, which are cells that have the microbicidal armory to destroy most pathogens. However, *M. tuberculosis* seems to have
evolved mechanisms to survive most of the macrophage-effector functions. Some of these mechanisms involve the inhibition of the phagosome-lysosome fusion, where the bacilli have been found to retain a macrophage protein, called tryptophane aspartate-containing coat protein (TACO), on the surface of the phagosome, preventing their delivery to the lysosome (Frataazzi et al., 1999), and to use complement receptors 1 and 3 for cell entry, which do not trigger oxidative burst (Schlesinger et al., 1990; Wright and Silverstein, 1983). Other mechanisms of survival include degradation of reactive oxygen intermediates by catalase and superoxide dismutase produced by the bacilli, inhibition of apoptosis in infected macrophages (Frataazzi et al., 1999), and down-regulation of some modulators of the host immunity such as interleukin 12 (IL-12) (Hickman et al., 2002; Nau et al., 2002), major histocompatibility complex (MHC) class II (Noss et al., 2000), and interferon γ (IFN-γ), known to mediate activation of macrophages (Ting et al., 1999).

After *M. tuberculosis* has entered the lungs, one of the four potential fates might occur (Schluger and Rom, 1998):

1) The initial host response can be completely effective in the killing and elimination of the bacilli, such that these individuals have no chance to develop TB.

2) The bacilli can grow and multiply immediately after infection, causing clinical disease (primary TB).

3) The bacilli may become dormant and never cause disease at all, resulting in a latent infection that is manifested only as positive tuberculin skin test results;
Figure 1: Different outcome of *M. tuberculosis* infection and underlying immune mechanisms. *M. tuberculosis* enters the host within inhaled droplets.
4) The dormant bacilli can eventually begin to grow, due to factors like Immunosuppression, with resultant clinical disease (reactivation TB).

2.3 History:

First credible understanding that tuberculosis might be due to infectious microorganisms was made in 1722 by Benjamin Martin of London, who proposed that the cause of tuberculosis was "animalcule or their seed inimicable to our Nature" that can be transmitted by "a Breath (a consumptive) emitted from his lungs that may be caught by a sound person" (Doetesch 1978; Castiglioni 1933). The tuberculosis appears to be as old as humanity itself. TB can be present in various forms, including one that attacks bone and causes skeletal deformities. Hard tissues like bone can be preserved for thousands of years, allowing almost certain identification of individuals with bone TB, who died more than 4000 years ago. Skeletal remains of prehistoric humans dating back to 8000BC, found in Germany have shown clear evidence of the disease.

More than a century after Laenmec's birth, Villemin performed experiments on rabbits, injecting infectious sputum and caseous material into healthy animals to produce disease. The studies conducted in 1868 and cited by major (1945), provided first convincing evidence of the infectious nature of tuberculosis. Gradually, the infectious nature of tuberculosis became more widely recognized. As early as 1699, Italy and later Spain enacted restrictive quarantine laws, while in Northern Europe; tuberculosis was not widely viewed as a public health problem.
The frequency of unearthed skeletons with apparent tuberculosis deformities in ancient Egypt suggests that the disease was common among that population. The discovery of similarly deformed bones in various Neolithic sites in Italy, Denmark and countries in the Middle East also indicates that TB was found throughout the world up to 4,000 years ago. (Smith, 2003). Ancient Hindu and Chinese writing have documented the presence of this disease.

The underdeveloped world including India is still suffering with high TB morbidity and mortality rates as shown by the following statistics. The incidence of TB ranges from less than 10 per 100,000 in North America to 100 to 300 per 100,000 in Asia and Western Russia to over 300 per 100,000 in Southern and Central Africa. There is one death from TB every 15 sec (Over two million per year) and eight million people develop TB every year. Without treatment, up to 60 percent of people with the disease will die (Kaye and Frieden, 1996).

Essentially all these cases are in the Third World (World Health Organization. 2002), reflecting the poverty and the lack of healthy living conditions and adequate medical care (Waaler, 2002). This global crisis is compounded by the emergence of multidrug resistance in countries like the former Soviet Union, South Africa, and India, where some antibiotics are available but are of inferior quality or are not used for a sufficient time to control the disease according to recommended regimens (Iseman, 1994: O’Brien, 2001).
Unlike many infectious diseases the epidemic wave of TB measures and Centuries-long epidemiological information though incomplete, reflects the incidence and prevalence of disease over a period of two to three centuries. The wave from of the tuberculosis epidemic occurs by natural selection of susceptible persons and runs its course in about 300 years. (William and Dutt. 2002).

In England, the presence of epidemic wave began in the 16th century and probably reached its peak about 1780 as a result of the industrial revolution and the growth of cities, which allowed the spread of disease from person to person. The epidemic then rapidly spread from England to other large-cities in Western Europe, reaching a peak in the early 1800s. In Eastern Europe the peaks came about 1870 and 1888 and by 1900 North American and South American epidemic waves had peaked. In the developing countries of Asia and Africa, the wave has not peaked yet. Thus, as a global phenomenon, the epidemic is declining in one geographic area while still rising or just reaching its peak in another.

Industrialization and overcrowding of cities can produce an epidemic of tuberculosis by bringing together large numbers of susceptible people and promoting transmission of *Mycobacterium tuberculosis* to new hosts.

TB morbidity and mortality rates due to TB steadily dropped during the 20th century in the developed world, aided by better public health practices and widespread use of the *M. bovis* BCG vaccine as well as the development of
antibiotics in the 1950s. This downward trend ended and the number of new cases started increasing in the mid-1980s. The major causes of this were increased homelessness and poverty in the developed world and the emergence of AIDS, with its destruction of the cell-mediated immune response in co-infected persons. Only by massive expenditures of funds and human resources mainly by directly monitored antibiotic delivery “miniepidemic” of new TB cases has been reversed in Europe and the United States (Frieden et al., 1995).

Thus, TB is caused by bacterial, but environmental factors play a major role, an idea that Rene Dubos clearly rearticulated 50 years ago (Dubos and Dubos, 1952). To Dubos, purely medical solutions alone would not work to cure and prevent TB. Unfortunately, the events of the last half of the 20th century have shown how prescient he was. The antibiotic era, begun by the discovery of streptomycin by Schatz and Waksman in the 1940s and its use to treat TB and followed by the introduction of many other antibiotics like isoniazid, rifampin, and pyrazinamide that are useful against TB, has not eliminated the disease (Ryan, 1992). Likewise, the widespread use of BCG, an attenuated vaccine strain produced by the sequential passage of a virulent M. bovis strain by Calmette and Guerin in Paris in the 1920s, has not lowered the incidence of TB in recent years (Andersen, 2002) and there is more TB today than ever before (Waaler, 2002).

2.3.1 NON TUBERCULOUS MYCOBACTERIA

More than 25 Mycobacterial species other than M. tuberculosis can be found in specimens from humans. Some of these species may have quite a high
likehood of being human pathogens. They include rapidly growing *M. fortuitum* and *M. cheloneae* and slowly growing *M. kansasii, M. avium*, *M. intracellulare*, *M. scrofulaceum, M. xenopi, M. malmoense, M. simiae*, *M. szulgdi, M. marinum, M. haemophilum* and *M. ulcerans*.

### 2.4 Features of *Mycobacterium Tuberculosis*

A characteristic feature of the *M. tuberculosis* includes its slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity. The generation time of *M. tuberculosis*, in synthetic medium or infected animals, is typically ~24 hours. This contributes to the chronic nature of the disease, imposes lengthy treatment regimens and represents a formidable obstacle for researchers. The state of dormancy in which the bacillus remains quiescent within infected tissue may reflect metabolic shutdown resulting from the action of a cell-mediated immune response that can contain but not eradicate the infection. As immunity wanes, through ageing or immune suppression, the dormant bacteria reactivate, causing an outbreak of disease often many decades after the initial infection. The molecular basis of dormancy and reactivation remains obscure but is expected to be genetically programmed and to involve intracellular signaling pathways.

#### 2.4.1 Growth

*M. tuberculosis* grows well on liquid and solid media such as: -
A. Liquid media

(i) Middlebrook 7H9 and 7H12B broth supplemented with 10% ADC (albumin dextrose and catalase) at 37° C.

(ii) Sautons media for 6-7 weeks at 37° C (Figure-2 a)

B. Solid media

(i) Middlebrook 7H10-7H11 agar supplemented with 10% OADC (Oleic acid, albumin dextrose and catalase) at 37° C

(ii) Lownstein Jenson (LJ) slants. (Figure –2 b)

Figure 2: Growth of M. tuberculosis culture on (a) liquid medium and (b) on solid medium.

2.4.2 Pigmentation

Depending on the pigment produced as well as rate of growth (Runyon et al., 1959) sub classifies the non-tuberculosis bacteria in four subgroups.

a. Sub Group1: Photomorphogens: These organisms grow slowly. Culture, when exposed to light turns yellow. e. g. M. Kansasii, M. Mannum, M. Simiae
b. **Sub Group 2: Scotochromogen**: These organisms grow slowly & form yellow pigment even in dark. When the culture is grown in light, the pigment is orange. e. g. *M. scrofulaceum, M. szulgai, M. gordonae*

c. **Sub Group 3: Nonchromogens**: Culture of these organisms is colorless & slightly pigmented; growth is slow and inhibited at 22° C but is common at 44° C. e. g *M. avium, M. intracellulare, M. ulcerans*

d. **Sub group 4: Rapid growers**: On primary culture growth may be slow, but in subcultures, growth may be evident in 2-3 days. Main organism in this sub group or category is *M. fortuitum, M. chelonae.*

### 2.4.3 Resistance

a. *Mycobacteria* can be killed at 60° C for 15-20 min.

b. Culture may be killed on direct sun light up to 2 hrs.

c. Bacilli may remain viable in droplet nuclei for 8-10 days

d. Bacilli in sputum may remain active for 20-30 min.

e. Culture may remain viable at Room temperature for 6-8 months and at -20 °C for 2 years.

### 2.4.4 Resistance to chemical disinfectants

a. Phenol 5%

b. Sulfuric acid 15%

c. Nitric acid 3%

d. Oxalic acid 5%

e. Sodium hypo chloride 4%

For 5 minutes, the organism can be destroyed by tincture of Iodine and 80% Ethanol in 2-10 min. However 80% ethanol is recommended.
2.4.5 Primary risk factor

a. Poverty
b. Malnutrition
c. Over crowding condition (Over population)
d. TB spreads more easily among family members living in the same house and breathing the same air.
e. It’s spread by aerosol droplets and causes irreversible lung destruction. If it escapes from the lung, it may cause systemic disease affecting many organs including bones, joints, liver, spleen, gastrointestinal tract and brain.

2.5 Epidemiology

Progress towards eradication of bovine tuberculosis in several countries has been impeded by a lack of precise understanding of epidemiological factors, such as the significance of inter-bovine transmission and the role of wild animals in maintaining disease, as well as other husbandry and environmental factors. Skuce and Neill (2003) reported that advances in strain genotyping methods now provide the potential to assist in identifying more accurately the sources of infection and major routes of transmission as well as outbreak contacts. Systematic investigation involving strain genotyping with appropriate interventions could probably elucidate more precisely the spread of bovine tuberculosis by identifying strain movement, to and/or from contiguous herds, spread within-herd, persistence on farm, recrudescence and re-introduction. In UK and Ireland, investigating further the geographical association between badger and cattle strains could accurately validate perceived associations
between badgers, other wildlife and tuberculous cattle. The direction of *M. bovis* transmission between wildlife and cattle may be inferred from temporal and geographically based information on prevalent strains involved. Additionally, finding of unusual *M. bovis* genotypes amongst prevalent strains in a particular area with unexpectedly identified reactor cattle could alert to fraudulent activity in cattle movement. Furthermore, *M. bovis* strains may differ in their immunogenicity and pathogenicity, both of which could affect tuberculosis detection. There is already some evidence that *M. bovis* strain type may influence skin test effectiveness (Goodchild *et al.*, 2003).

Established strain typing techniques such as restriction enzyme analysis (REA), (Collins, 1999) and restriction fragment length polymorphism (RFLP) typing (Van Soolingen, 2001) have been standardized and are routine procedures in several public health and veterinary research laboratories. However, these techniques are technically demanding and require expensive softwares for analysis and archiving of complex banding patterns. The achievement of inter-laboratory reproducibility is not trivial (Heersma *et al.*, 1998). Some genetic markers may lack discrimination with some members of the *M. tuberculosis* complex. For example, IS6110-RFLP analysis and spoligotyping (Kamerbeek *et al.*, 1997) are poorly discriminating for most *M. bovis* isolates, although spoligotyping does provide additional phylogenetic data.

In New Zealand, REA typing has provided a new insight into disease dynamics, demonstrating clustering of REA types in defined areas. Possums, other wildlife and farmed animals in the same area were often infected with
the same REA type. REA typing has been used to include or exclude possible sources of infection in specific herds and it has demonstrated clearly whether infection in farmed animals has come from the infected local wildlife reservoir, or from infected cattle and deer, brought onto those premises. REA strain typing has been used to influence the level of herd testing or wildlife control in specific areas and is now considered an integral component in local TB control schemes (Collins, 1999).

The genome sequencing projects have disclosed several different classes of genetic marker, including insertion sequences, insertion/deletion events, tandem repeat loci and point mutations (single nucleotide polymorphisms), all of which are now being exploited in molecular epidemiology studies, in an attempt to develop more convenient strain typing techniques for *M. bovis*, that are amenable to uncomplicated analysis, intuitive nomenclature and reliable inter-laboratory comparison. Identification of repetitive DNA, such as variable number tandem repeats (VNTRs) in the genome sequences of *M. tuberculosis* strains H37Rv and CDC 1551 (Cole *et al.*, 1998) and *M. bovis* AF2122/97 has been exploited recently in strain typing and high throughput, highly discriminating and reproducible assays have been configured (Roring *et al.*, 2004). For local applications, it is important to characterize prevalent strains and the choice and combination of VNTR markers, which are most informative in a given location, needs to be determined empirically.

A striking feature of applying both spoligotyping and the higher-resolution VNTR assay to surveillance, at least in the UK and Ireland, is that *M. bovis* strain appear to cluster in defined geographical areas (Smith *et al.*, 2003)
suggesting that sources of infection are stable and local. Combining spatial
data with hypothetical phylogenies based on spoligotyping and VNTRs have
suggested a series of 'clonal expansions', where a particular VNTR genotype
increases dramatically in frequency. This is either via selection or ecological
opportunity but most likely due to simple invasion, rather than resulting from
enhanced adaptation to wildlife and / or cattle. These techniques have also
been used to establish, from cattle movement data, the probability of infection
being bought-in or acquired on arrival (Durr et al., 2004). Strain typing has
already provided evidence of within-herd spread and the introduction of
infection through long-range cattle movements.

2.6 Cell structure and Metabolism

*Mycobacteria* are rod-shaped, Gram-positive aerobes, or facultative
anaerobes. As deduced from its genome, *M. tuberculosis* has the
potential to manufacture all of the machinery necessary to synthesize all
of its essential vitamins, amino acids, and enzyme co-factors. On the
other hand, the inability to culture *Mycobacterium leprae*, suggests that
it has lost many of its metabolic capabilities, and is now an obligate
parasite, dependent on its host for most of its nutritional needs (Cole et
al., 1998). This goes in accordance with its severely degenerated
genome additionally *M. tuberculosis* has an unusual cell wall, with an
additional layer beyond the peptidoglycan layer, which is rich in unusual
lipids, glycolipids, and polysaccharides.
2.7 Taxonomic Status

The generic name *Mycobacterium* (Fungus - Bacterium) was first coined by Lehmann & Newmann (1996) in the 1st edition of their book *Atlas of Bacteriology*, because of the mould like pellicular growth of the tubercle bacillus on liquid media. The variations of properties within the genus *Mycobacterium* is enormous and is reflected in the range of virulence, habitat, rate of growth, nutritional requirements and antigenicity.

![Figure 3: Electron micrograph of *M. tuberculosis*. Courtesy of the Institut Pasteur image library.](image)

Many of the unique characteristics of *Mycobacteria* are found to be in their very complex lipid rich cell walls. *Mycobacteria* are gram-positive, aerobic, non-sporing & non-motile (Figure-3). These do not form capsules but have the lipids termed mycosides. *Mycobacteria* are important group of organisms, because many of them are pathogenic to Human as well as animals. *Mycobacteria* are gram positive, usually acid and alcohol fast at some stage of growth, they do not form capsules, endospores or conidia, rarely exhibit grossly visible aerial hyphae, produce acid from sugar by oxidation & with exception of those that do not grow *In vitro* can be divided into rapid and slow growing strain. The original description of the genus was based mainly on morphological & staining properties - features that are considered sufficiently
diagnostic to distinguish *Mycobacteria* from Actinomycetes (Skerman *et al.*, 1980).

Further publications of newly recognized species, primarily of rapid growers have now brought the number to 54. Of these 54, 17 species are pathogenic & others are non-pathogenic. Members of genus *Mycobacterium* exhibit a great deal of variability with regard to growth rate, disease causing capability and physiology. Different *Mycobacterial* species have been described and classified on the basis of rate of growth, chromogenecity etc. Although *Mycobacteria* were not formerly divided into subgenera, it is almost essential to consider it in two major categories. With the exception of *Mycobacterium leprae*, which is still to be cultivated *in vitro*, *Mycobacteria* can be assigned into two groups primarily on the basis of growth rates. The so called rapid growers include strains of those species which under optimal conditions of nutrition & temperature produce colonies within seven days, whereas, their slow growing counterparts require an additional view or more to form such colonies under such conditions. The optimum temperature of both species ranges from 28-45 °C.

### 2.8 *Mycobacterial* genome

*M. tuberculosis* H$_{37}$Rv genome comprises of 4,411,529 base pairs, and contains around 4000 genes (3924 predicted protein coding genes). The genome has a very high guanine + cytosine content (65.6 %) that is reflected in the biased amino acid content of the proteins. There is a significant
preference for the amino acids Ala, Gly, Pro, Arg and Trp, which are all encoded by G+C rich codons, and a comparative reduction in the use of amino acids encoded by A+ T rich codons such as Asn, Ile, Lys, Phe and Tyr. *M. tuberculosis* radically differs from other bacteria in a very large portion of its coding capacity is devoted to the enzymes involved in lipogenesis and lipolysis. In total there are ~250 distinct enzymes involved in fatty acid metabolism in *M. tuberculosis* compared with only 50 in *E. coli* (Cole *et al.*, 1998).

### 2.9 TRANSMISSION

The principal risk for acquiring infection with *Mycobacterium tuberculosis* is breathing (Bloom and Murray 1992). The classic studies of Wells (1955) and his student Riley (Riley and O'Grady, 1961) established beyond doubt that infectious particles containing *M. tuberculosis* were emitted in coughs and sneezes and even while speaking, a sneeze may contain over a million particles with diameters of less than 100µm, the mean being 10 µm. This particle from droplet nuclei in which evaporation continues until the vapor pressure of the droplet equals the atmospheric pressure. The droplet nucleus is very stable, settles very slowly (about 12mm/min), and remains suspended in the air for long periods, A 10 µm droplet nucleus may carry perhaps 3 to 10 tubercle bacilli. Dust associated particles may also carry *M. tuberculosis*. These particles are larger than droplets, but they can be transiently resuspended by air convection and may serve as a reservoir for infectious bacilli. Infection can take place by ingestion of tubercle bacilli but is about 10,000 fold less effective than inhalation of droplets on transmitting
tuberculosis; probably tubercle bacilli are very sensitive to gastric acid (Gaudier and Gernez-Rieux, 1962). The number and concentration of bacilli present on the source case estimated to be between $10^2$ to $10^3$ in solid or nodular lesions but of the order of $10^2$ to $10^3$ in cavitary lesions (Canetti, 1965), are major variables in transmission, duration of exposure and the aerodynamics of the exhaled particles.

Thus lung is major portal of entry in the majority of cases of tuberculosis (Glassroth et al., 1980s; Mayock and Mac, 1976). Pulmonary TB has been variously described as consumption. The terms indicating the severe wasting and the coughing of blood associated with later stages of the disease. Tuberculosis also can develop in the central nervous system, in which case meningitis is the predominant from of the disease and also in the urogenital tract, the digestive system and cutaneously in the from named lupus vulgaris. The incidence of these various extrapulmonary forms of tuberculosis varies from country to country, such that on the average between 1964 and 1989, 20 percent of the 20,000 new cases of TB in the United States were extrapulmonary while 5 to 10 percent of the approximately seven million new cases each year in the developing countries were extrapulmonary (Talavera et al., 2001).

This distribution also can be affected by origin of the individuals within a country. In one study of TB patients in England, 20 percent of patients of European origin had extrapulmonary TB, of which lymph node, bone and joint and genitourinary involvement accounted for almost 90 percent. Of patients whose origin was on the Indian subcontinent, 45 percent had extrapulmonary
tuberculosis and 60 percent of these sites of infection were in lymph nodes and in bones and joints (Yates, and Grange, 1993). Autopsies of deceased human immunodeficiency virus (HIV) negative TB patient in another study in New York City showed that 68 percent had extrapulmonary TB whose lesions were widely and randomly distributed throughout the body with no apparent predilection for a limited number of sites as noted in the English study (Jagirdar and ZagZag, 1996).

Tuberculosis usually affects the lungs although in up to one-third of cases, other organs are involved. In order of frequency, the extrapulmonary sites most commonly involved in tuberculosis are the lymph nodes, pleura, genitourinary tract, bones and joints, meningitis and peritoneum. However, virtually all organ systems may be affected as a result of hematogenous dissemination in HIV-infected individuals, extrapulmonary tuberculosis is seen more commonly today than in the past.

The risk of developing disease after being infected depends largely on endogenous factors, such as the individual’s innate susceptibility to disease and level of function of cell-mediated immunity.

Age is an important determination of the risk of disease after infection. Among infected persons, the incidence of tuberculosis is highest during late adolescence and early adulthood, the reasons are unclear.
The incidence among women peak at 25 to 34 years of age group rates among women are usually higher than those among men, while at older ages the opposite is true. The risk may increase in the elderly, possibly because of waning immunity and co morbidity.

A variety of diseases favor the development of active tuberculosis. The most potent risk factor for tuberculosis among infected individuals is clearly HIV co-infection, which suppresses cellular immunity. The risk that latent *M. tuberculosis* infection will precede to active disease is directly related to the patient’s degree of Immunosuppression. In a study of HIV-infected, PPD-positive person, this risk varied from 2.6 to 13.3 cases per 100 person–years and depend upon the CD4+ cell count. This risk of developing tuberculosis is significantly greater among HIV-infected than among HIV- uninfected hosts. Other conditions known to increase the risk of active tuberculosis among person infected with tubercle bacilli include silicosis, lymphoma, leukemia and other malignant neoplasm’s, hemophilia, chronic renal failure and hemodialysis, insulin-dependent diabetes mellitus, immunosuppressive treatment, including that administered for solid-organ transplantation and condition associated with malnutrition such as gastrectomy and jejunoileal byepass surgery. Finally, the presence of old self-healed fibrotic tuberculosis lesion constitutes a serious risk of active disease. (Handbook of Harrison’s, 2001).

In modern times, the respiratory route of exposure initiates most TB infections now that milk products are generally TB cases were pulmonary (Hopewell, 1994). Thus, the different forms of the disease discussed above usually arise from dissemination of the bacilli from infected lungs. TB in many cases follows
a general pattern as described by Wallgren, who divided the progression and resolution of the disease into four stages (Wallgren, 1948). In the first stage, dating from 3 to 8 weeks after \textit{M. tuberculosis} contained in inhaled aerosols becomes implanted in alveoli the bacteria are disseminated by the lymphatic circulation to regional lymph nodes in the lung, forming the so-called primary or Ghon complex. At this time, conversion to tuberculin reactivity occurs. The second stage, lasting about 3 months, is marked by hematogenous circulation of bacteria to many organs including other parts of the lung; at this time in some individuals, acute and sometimes fatal disease can occur in the form of tuberculosis meningitis (disseminated) tuberculosis. Pleurisy or inflammation of the pleural surface can occur during the third stage, lasting 3 to 7 months and causing severe chest pain, but this stage can be delayed for up to 2 years. It is thought that this condition is caused by either hematogenous dissemination or the release of bacteria into the pleural space from subpleural concentrations of bacteria in the lung. The free bacteria or their components are thought to interact with sensitized CD4 T-lymphocytes that are attracted and then proliferate and release inflammatory cytokines (Kamholz, 1996). The last stage or resolution of the primary complex, where the disease does not progress, may take up to 3 years. In this stage, more slowly developing extrapulmonary lesions, e.g., those in bones and joints, frequently presenting as chronic back pain, can appear in some individuals. However, most humans who are infected with TB do not exhibit progression of the disease.
One-third of exposed HIV-negative individuals become infected, and of this number 3 to 5% develop TB in the first year. An additional 3 to 5% of those infected develop TB later in their lives. It is thought that most adult TB in non-HIV-infected patients is caused by reactivation of preexisting infection (Garay, 1996). HIV-positive persons infected with *M. tuberculosis* have a 50% chance of developing reactivation (post primary) TB at some time in their lives. These individuals and others who are immunosuppressed can also be newly infected with *M. tuberculosis* and in many cases show rapid progression to active disease (Garay, 1996 b). Adult TB, whether resulting from activation or new infection in HIV-infected patients, is almost always pulmonary and is associated with differing degrees of lung involvement and damage, notably necrosis, cavitation and bleeding (Jagirdar and ZagZag, 1996).

As discussed above, the interaction of *M. tuberculosis* with the human host begins when droplet nuclei containing microorganisms from infectious patients are inhaled. While the majority of inhaled bacilli are trapped in the upper airways and expelled by ciliated mucosal cells, a fraction (usually fewer than 10%) reaches the alveoli. It is also possible that bacteria can be initially ingested by epithelial type-II pneumocytes. This cell type is found in greater numbers than macrophages in alveoli and *M. tuberculosis* can infect and grow in these pneumocytes *ex vivo* (Bermudez and Goodman, 1996; Mehta *et al*., 1996). In addition, dendritic cells play a very important role in the early stages of infection since they are much better antigen presenters than are macrophages and presumably play a key role in activating T-cells with specific *M. tuberculosis* antigens (Bodnar *et al*., 2001; Gonzalez-Juarrero *et
Since dendritic cells are of migrating nature unlike differentiated macrophages (Lipscomb and Masten, 2002), they also may play an important role in dissemination of *M. tuberculosis*. Invasion of macrophages by *Mycobacteria* may result in part from association of C2a with the bacterial cell wall followed by C3b opsonization of the bacteria and recognition by the macrophages. The bacteria are phagocytosed in a process that is initiated by bacterial contact with macrophage mannose and / or complement receptors (Schlesinger, 1993). Surfactant protein A, a glycoprotein found on alveolar surfaces can enhance the binding and uptake of *M. tuberculosis* by up regulating mannose receptor activity (Gaynor *et al.*, 1995).

On the other hand, surfactant protein D, similarly located in alveoli, inhibits phagocytosis of *M tuberculosis* by blocking mannosyl oligosaccharide residues on the bacterial cell surface (Ferguson *et al.*, 1999), and it is proposed that this prevents *M. tuberculosis* interaction with mannose receptors on the macrophage cell surface. Cholesterol in cell plasma membranes is thought to be important for this process, since removal of this steroid from human neutrophils decreases the phagocytosis of *M. kansasii* (Peyron *et al.*, 2000) and similar depletion experiments prevented the entry of *M. bovis* BCG into mouse macrophages (Gatfield and Pieters, 2000). The human toll-like receptor 2 (TLR2) also plays a role in *M. tuberculosis* uptake (Noss *et al.*, 2001).

On entry into a host macrophage, *M. tuberculosis* and other intracellular pathogens initially reside in an endocytic vacuole called the phagosome. If the normal phagosomal maturation cycle occurs, i. e., phagosome-lysosome
fusion, these bacteria can encounter a hostile environment that includes acidic pH, reactive oxygen intermediates (ROI), lysosomal enzymes, and toxic peptides. Reactive nitrogen intermediates (RNIs) produced by activated mouse macrophages are major elements in antimicrobial activity (Nathan and Hibbs, 1991), and mice with mutations in the genes encoding the macrophage-localized cytokine inducible nitric oxide synthase genes are more susceptible to various pathogens, including *Leishmania major* (Wei, 1995), *Listeria monocytogenes* (MacMicking, 1995), and *M. tuberculosis* (MacMicking, 1997). The *M. tuberculosis* result is consistent with the results of other experiments showing that RNIs are the most significant weapon against virulent *Mycobacteria* in mouse macrophages (Chan *et al.*, 1995; Chan *et al.*, 1992) and the observation that resistance to RNIs among various strains of *M. tuberculosis* correlates with virulence (Chan *et al.*, 1995; Chan *et al.*, 1992; O'Brien *et al.*, 1994). The presence of RNIs in human macrophages and their potential role in disease has been the subject of controversy, but the alveolar macrophages of majority of TB-infected patients exhibit iNOS activity (Nicholson *et al.*, 1996).

**2.10 Survival of *M. tuberculosis***

Accumulation evidence suggests that *M. tuberculosis* enters macrophages via specific binding to cell surface molecule of phagocytes. It has been reported that the tubercle bacillus can bind directly to the mannose receptor via the cell wall-associated, mannosylated glycolipid LAM or indirectly via complement receptors of the integrin family (CR1, CR3) or Fc receptors. Phagocytosis, triggered by engaging certain cell surface molecules such as the Fc receptor,
stimulates the production of ROI via activation of the oxidative burst. Experimental data indicate that *M. tuberculosis* can interfere with the toxic effect of ROI by various mechanisms. First, various *Mycobacterial* compounds including glycolipids (GL), sulfatides (ST) and LAM can down regulate the oxidative cytotoxic mechanism. Second uptake via CR1 bypasses activation of the respiratory burst. Cytokine-activated macrophages produce RNI that, at least in the mouse system, mediate potent antiMycobacterial activity. The acidic condition of the phagolysosomal vacuole can be conductive to the toxic effect of RNI. However, NH$_4^+$ production by *M. tuberculosis* may attenuate the potency of the L-arginine-dependent antiMycobacterial mechanism and that of lysosomal enzymes, which operate best at an acidic pH. In addition, *Mycobacterial* products such as sulfatides and NH$_4^+$ may interfere with phagolysosomal fusion. Finally, the tubercle bacillus may evade the highly toxic environment by escaping into the cytoplasm via the production of hemolysin.

2.11 Pathogenesis & Immunology

Pathogenicity for human for some of the following bacteria is questionable: *M. gordonae, M. asisticum, M. terrae, M. triviale, M. nonchromogenicum, M. gastri, M. flavescens* and *M. phlei*. These species may be found in sputum specimens, except for *M. marinum, M. haemophilum and M. ulcerans*, which are typical of specimens from skin lesions. Any of these species, can be found in blood specimens obtained from HIV-infected individuals, although *M. avium* is most often isolated. Since *M. tuberculosis* can also be isolated from blood specimens, one must give special attention to the possibility of mixed culture.
The frequency of finding more than one *Mycobacterial* species in sputum specimens from non-HIV patients is increasing as well.

2.11.1 Pulmonary TB

The most common clinical manifestation of TB is the pulmonary disease. After inhalation, the bacilli initiate small lesions in the lower respiratory tract. These lesions frequently heal to form tiny tubercle, which are too small to be seen by x-rays but may continue to harbor the bacilli indefinitely. In other cases, replication of the bacilli continues and the lesions expand and undergo caseation necrosis, which will destroy the normal tissue and leave the necrotic tissue in a semisolid, "cheesy" state. Caseation necrosis may eventually heal and become infiltrated with fibrous tissue and calcium deposits, or may continue to expand leaving cavities in the lungs (Gebbardt et al., 1996).

3.11.2 Extra pulmonary TB

Extrapulmonary TB is more common in children and in HIV-infected individuals (Shafer and Edlin, 1996). In extrapulmonary TB, the tubercle bacilli may spread through the bloodstream from the lesions in the lungs into other organs such as, bones and joints particularly the spine (Okuyama et al., 1996), kidneys and genital tract causing genitourinary TB (Gorse and Belshe, 1985), or the central nervous system causing TB meningitis (Thwaites et al., 2000). TB meningitis is fatal in almost all cases without treatment, therefore prompt identification and chemotherapy are crucial to prevent serious neurological sequels.
Another clinical manifestation of extrapulmonary TB is disseminated TB, which is defined as involvement of many organs simultaneously, and can occur as result of a primary progressive disease or reactivation of the latent infection (Hill et al., 1991).

The pathogenesis of tuberculosis described series of battles between the host and the parasite; the weapons of the host are - 1) activated macrophage- to kill the tubercle bacilli that it ingests. 2) Inhibitory- potential to stop the intracellular growth of bacilli in a non-activated macrophage by killing the macrophage, thereby transforming a favorable intracellular environment in to the inhibitory environment of solid caseous tissue.

The weapons of tubercle bacillus are (1) Logarithmic multiplication ability within non-activated macrophages, i. e., within the monocytes that recently immigrated into the tissues’ local sites of the infections. (2) Extracellular multiplication ability, often reaching tremendous numbers, in the liquefied caseous focus which often forms activity.

Despite the strong host weapons against tubercle bacillus the host is vulnerable due to (1) the non-activated macrophages, which provide a favorable intracellular environment are bacillary growth and (2) material which is the only menstruum that supports the extracellular growth of the bacillus.

The vulnerability of the bacillus are- (1) an inability to survive within a fully activated macrophage. (2) An inability to multiply in solid caseous tissue.

Cell mediated immunity (CMI), aquired cellular resistance (ACR), and
Delayed-type hypersensitivity (DTH) are immunological responses produced by the host that play key role in the pathogenesis of tuberculosis. The tubercle bacillus apparently cannot injure host tissue until these immune responses develop.

Usually the control of the infection at some point during various stages of disease progression in the sensitive host, the following stages have been marked- strain of M.TB prior exposure, vaccination, Infectious dose, Immune status of the host.

2.11.2 Stages of Tuberculosis infection

Stage-1.

There is no bacillary growth. The bacillus growth, the bacillus is usually destroyed or inhibited by the mature resident AM that ingests it. If the bacillus is not destroyed, it grows (i.e. multiplies) and eventually destroys the AM.

Stage-2.

This begins after 7-21 days of initial infection. Mycobacterium Multiples logarithmically with the immature unactivated macrophages of the developing lesion (now called a tubercle). This ultimately leads to burst of the macrophages. Other macrophages begin to extravasate from peripheral blood. These macrophages also phagocytose M. TB, but they are also inactivated and hence cannot destroy M. TB.

Stage-3.

The number of viable bacilli becomes stationary because there growth is inhibited by the immune response. This immune response has two components- (a) Cell-mediated immunity (CMI), i.e., activated macrophages
and (b) delayed-type hypersensitivity (DTH), i.e. caseous necrosis. Cell-mediated immunity is critical at this early stage which confers partial protection against *M. tuberculosis*, while humoral immunity has no defined role in protection. Two types of cells are essential macrophages, which directly phagocytize tubercle bacilli and T-cells (mainly CD4+ lymphocytes), which induce protection through the protection of lymphokines. At this stage lymphocytes begin to infiltrate. The lymphocytes, specifically T-cells, recognize processed and presented M. TB antigen in content of molecule. Coincident with the appearance of immunity, DTH to *M. tuberculosis* develops. This reactivity is the basis of the PPD skin test, currently the only test that reliably detects *M. tuberculosis* infection in persons without symptoms. The cellular mechanisms responsible for PPD reactivity are related mainly to previously sensitized CD4+ lymphocytes, which are attracted to the skin-test site. There, they proliferate and produce cytokines.

Antigen processing and presentation by macrophages to T-lymphocytes result in proliferation of CD4+ lymphocytes, which are crucial to the host’s defense against *M. tuberculosis*. Qualitative and quantitative defects of CD4+ T cells explain the inability of HIV-infected individuals to contain *Mycobacterial* proliferation. Reactive CD4+ lymphocytes produce cytokines of the T\textsubscript{h}1 pattern and participate in MHC class II-restricted killing of cells infected with *M. tuberculosis*. T\textsubscript{h}1 CD4+ cells produce interferon-\alpha (IFN-\alpha) and IL-2 and promote cell-mediated immunity. T\textsubscript{h}2 cells produce IL-4, IL-5, and IL-10 and promote humoral immunity. The interplay of these various cytokines and their cross-regulation determine the host’s response. The role of cytokines in
promoting intracellular killing of *Mycobacteria* has not been entirely elucidated. IFN-α may induce release of nitric oxide, and TNF-α also seems to be important. Finally, the role of other cells, such as natural killer (NK) cells, "double-negative" CD4+, CD8+ cells, and T cells, in protective immunity remains unclear.

It is at this stage that the individual becomes tuberculin-positive. This positive tuberculin reaction is the result of the host developing a vigorous cell mediated immune (CMI) response. A CMI response must be mounted to control an M. TB infection. An antibody-mediated immunity (AMI) will not aid in the control of a M. TB. Infection because M. TB is intracellular and if extracellular, it is resistant to complement killing due to the high lipid concentration in its cell wall.

Although, CMI response is necessary to control *M. tuberculosis* infection. It is also responsible for much of the pathology associated with tuberculosis. Activated macrophages may release lytic enzymes and reactive intermediates that facilitate the development of immune pathology. Activated macrophages and T-cells also secrete cytokines that may also play a role in the development of immune pathology, including Interleukin 1 (IL-1), tumor necrosis factor (TNF), and gamma IFN, contributes to the killing of *Mycobacteria*, the formation of granulomas and a number of systemic effects such as fever and weight loss. It is at this stage that tubercle formation begins. The center of the tubercle is characterized by 'caseation necrosis' meaning semi-solid or 'cheesy' consistency M. TB. Cannot multiply within
these tubercles because of the low pH and anoxic environment. M. TB can however, persist within these tubercles for extended periods. The disease is arrested at this stage if the activated macrophage population predominates.

**In next stages** many activated macrophages can be found surrounding the tubercles, many other macrophages present remain unactivated or poorly activated. M. TB uses these macrophages to replicate and hence the tubercle grows. The growing tubercle may invade a bronchus. If this happens, M.TB infection can spread to other parts of the lung. Similarly the tubercle may invade an artery or other blood supply line. The hematogenous spread of M.TB may result in extrapulmonary tuberculosis otherwise known as milliary tuberculosis. The name "milliary" is derived from the fact that metastasizing tubercles are about the same size as a millet seed, a grain commonly grown in Africa.

The secondary lesions caused by milliary TB can occur at almost any anatomical location, but usually involve the genitourinary system, bones, joints, lymph nodes, and peritoneum. These lesions are of two types:

1. **Exudative lesions** result from the accumulation of PMN's around M. TB. Here the bacteria replicate with virtually no resistance. This situation gives rise to the formation of a "soft tubercle".

2. **Productive or granulomatous lesions** occur when the host becomes hypersensitive to tuberculoproteins. This situation gives rise to the formation of a "hard tubercle".
In the last stage of the liquefaction the bacillus evades host defenses. Reasons leading to liquefaction of caseous centers of tuberculosis remains are still unknown. This liquid is very conducive to M.TB growth and hence the organism begins to rapidly multiply extracellularly. After some time, the large antigen load causes the walls of nearby bronchi to become necrotic and rupture. This result in cavity formation, the activity due to the tissue destruction as a result of a delayed-type hypersensitivity (DTH) reaction to various bacillary antigens. The bacilli enter the bronchial tree and then other parts of the lung and the outside environment. Arrestment of the disease at this stage depends on whether the antigenic load (off both the bacilli and their products) remains small enough for the host to cope with only a very small percent of M.TB infections resulting in the formation of disease and even a smaller percentage of M. TB infection progress to an advanced stage. Usually the host will begin to control the infection at some point. When the primary lesion heals, it becomes fibrous and calcifies. When this happens, the lesion is referred to as the Ghon complex. Depending on the size and severity, the Ghon complex may never subside. Typically the Ghon complex is readily visible upon chest X-ray.

2.11.2.1 Immune response to TB

Innate immune response

Recent immunological and genetic studies have corroborated the longstanding notion that, innate immunity is relevant in the host defense against *M. tuberculosis*. 
The uptake of *M. tuberculosis* by alveolar macrophages represents the first step in the innate host defense against TB. This initial interaction is mediated by cellular receptors such as complement receptors, mannose receptors, surfactant receptors, and scavenger receptors (Chan *et al.*, 1992; Downing *et al.*, 1995; Flesch and Kaufmann, 1988; Gaynor *et al.*, 1995; Schlesinger *et al.*, 1993). Most recently, attention has been focused on the role of toll-like receptors (TLRs) in mediating the uptake of *Mycobacteria* by macrophages.

Specifically, the role of TLR2 and TLR4 in sensing *Mycobacteria* and promoting anti-*Mycobacterial* responses has been demonstrated in several studies. *In vivo* studies using TLR2 or TLR4 deficient mice have shown that these mice are more susceptible to *Mycobacteria* infection than wild-type mice (Quesniaux *et al.*, 2004).

Furthermore, *in vitro* studies using a human macrophage-like cell line have demonstrated that activation of TLRs by lipoproteins contained within the *M. tuberculosis* cell wall induces production of IL-12, an important pro-inflammatory cytokine in the host response against TB (Brightbill *et al.*, 1999). In addition, these studies showed that TLR-mediated IL-12 production also resulted in increased production of nitric oxide synthetase and nitric oxide, which are important for the intracellular killing of *Mycobacteria*.

Thus, TLRs contribute to the innate immunity by detecting *Mycobacteria*-associated molecular patterns and mediating the secretion of anti-*Mycobacterial* effector molecules. However, TLRs can also influence the
specific immunity by upregulation of immunomodulatory molecules supporting the development of pro-inflammatory responses (Schluger, 2001).

2.11.2.2 Specific immune response

The specific immune response to *M. tuberculosis* in the lungs is complex and involves multiple mechanisms. T cells are believed to be essential in the protective immune response against TB, and the interaction of T cells with macrophages is critical for the control of the infection. The production of inflammatory cytokines and chemokines, induced by ingestion of *M. tuberculosis* by alveolar macrophages (Means *et al.*, 1999), leads to the migration of monocyte-derived macrophages and dendritic cells to the site of infection. The dendritic cells that engulf *Mycobacteria* mature and migrate to regional lymph nodes (Bodnar *et al.*, 2001; Henderson *et al.*, 1997; Hertz *et al.*, 2001), where then T cells are primed against *Mycobacteria* antigens. Primed T cells expand and migrate to the site of infection in the lungs, presumably due to the upregulation of local adhesion molecules and chemokines. The migration of macrophages and T cells to the site of infection results in formation of a granuloma (Figure- 4), which also comprises other cells such as B cells, dendritic cells, endothelial cells, fibroblasts and probably stromal cells (Gonzalez-Juarrero *et al.*, 2001).

The granuloma functions as an immune microenvironment to facilitate interactions between T cells and macrophages. In addition to providing a framework for these cells, granulomas serve to wall off *Mycobacteria* from the rest of the lungs, limiting the dissemination of the infection. However,
depending on the cellular composition and on the cytokine and chemokine-secreting profile, granulomas can also be associated with pathology or at least lack of adequate containment of bacillary multiplication (Saunders and Cooper, 2000).

2.11.3 Role of CD4, CD8 and γδ T cells in the response against *M. tuberculosis* and latent infection

2.11.3.1: CD4+ T cells

CD4+ T cells play a central role in the immune response against *M. tuberculosis*. Peptide antigens from *Mycobacteria*, degraded in the phagolysosomal compartment and complexed with the MHC class II molecules are recognized by CD4+ T cells, resulting in their activation (Davis and Bjorkman, 1988). The main function of CD4+ T cells in immunity to TB is thought to be the production of cytokines, specifically IFN-γ, which is critical for macrophage activation and the subsequent induction of microbicidal mechanisms (Flesch and Kaufmann, 1990). The critical role of IFN-γ in the control of *Mycobacteria* infections has been demonstrated in animal models. Experimentally, mice deficient in IFN-γ or in IL-12, a critical cytokine in the induction of IFN-γ production, were highly susceptible to challenge with *M. tuberculosis* (Cooper et al., 1993; Cooper et al., 1997). In addition, studies in humans have shown that patients with IFN-γ receptor deficiency presented disseminated infection with *M. bovis* BCG and/or environmental *Mycobacteria*, which resulted in the death of about half of the patients and required continuous anti-*Mycobacterial* treatment in the survivors (Abel et al., 2002). CD4+ T cells can also contribute to the control of acute *Mycobacteria* infections through IFN-γ independent mechanisms. This has been
demonstrated in a variety of experimental models using antibody depletion and mouse strains deficient in either CD4 or MHC class II molecules (Caruso et al., 1999; Scanga et al., 2000). In mice deficient in CD4 or MHC class II molecules, the levels of IFN-γ were significantly diminished very early during infection, but later the IFN-γ production was similar to that seen in wild type mice. However, deficient mice were not rescued by this later production of IFN-γ, and succumbed to the infection. IFN-γ independent mechanisms of action of CD4⁺ T cells may also include a cytolytic function of these cells, as has been shown in murine models (Izzo and North, 1992) as well as in humans (Tan et al., 1997).

Further evidence of the importance of CD4⁺ T cells in the control of TB in humans is obtained from studies of the clinical course of co-infection with HIV. Depletion of CD4⁺ T cells during HIV infection dramatically increases the susceptibility to primary and reactivation TB (Havlir and Barnes, 1999; Jones et al., 1993).

2.11.3.2 CD8⁺ T cells

Despite the intraphagosomal location of *M. tuberculosis*, it is now recognized that CD8⁺ T cells, restricted either by MHC class I or CD1 molecules, participate in a successful anti-*Mycobacterial* immune response. In contrast to the peptide epitopes presented by the MHC molecules, CD1 molecules present lipids or glycolipids to T cells (Porcelli and Modlin, 1999).
Experimentally, mice deficient in β2-microglobulin, a component of both MHC class I and non-classical MHC class 1b molecules were found to be more susceptible to infection with *M. tuberculosis* than wild type mice (Flynn et al., 1992). Similarly, increased susceptibility to *Mycobacterial* infections has been seen in mice deficient in transporters associated with antigen processing (TAP) molecules, which transport peptides from the cytosol to the endoplasmic reticulum for loading into MHC class I molecules (Behar et al., 1999; Sousa et al., 2000). In addition to these studies, vaccination of mice with DNA plasmids expressing *Mycobacterial* antigens were also shown to induce antigen-specific CD8+ CTL, which conferred protection against challenge with *M. tuberculosis* (Smith and Dockrell, 2000). Despite all the experimental findings confirming the role of CD8+ T cells in the control of TB, it still remains unclear how phagosomically derived antigens interact with the MHC class I processing machinery.

CD8+ T cells appear to have two major functions in TB immunity, lysis of infected cells and production of cytokines, mainly IFN-γ. The relative contribution of these functions is unknown. It has been shown that CD8+ T cells from the lungs of infected mice are primed to produce IFN-γ, upon T cell receptor (TCR) interaction with *M. tuberculosis* infected dendritic cells (Serbina and Flynn, 1999). However, unlike CD4+ T cells spontaneous *ex vivo* production of IFN-γ by CD8+ T cells is very low, suggesting that the production of this cytokine by CD8+ T cells in the lungs is limited (Serbina and Flynn, 1999). Evidence for a more direct role of CD8+ T cells come from studies showing lysis of infected human macrophages and dendritic cells by CD1 and
MHC class I restricted CD8+ T cells specific for *M. tuberculosis*, resulting in reduced numbers of intracellular bacteria (Cho *et al.*, 2000; Stenger *et al.*, 1997). The killing of the intracellular bacteria was shown to be perforin-dependent (Stenger *et al.*, 1997). Perforin was required to form a pore, but the molecule responsible for the killing of the intracellular bacteria was granulysin, another cytotoxic granule protein (Stenger *et al.*, 1998).

2.11.3.3: γδ+ T cells

A large amount of evidence from human and animal studies suggests that, γδ+ T cells play a significant role in the host response to TB (Boom, 1996). It is generally believed that these cells are involved in primary immune defense. Indeed, a recent study reported that γδ+ T cells accumulated in the lungs of BCG-infected mice three weeks earlier than antigen-specific αβ+ T cells, suggesting that γδ+ T cells in the lungs might help to control *Mycobacterial* infection during the period between the innate and adaptive immunity.

Additionally, results suggested that γδ+ T cells might also play an important regulatory role in the subsequent onset of αβ+ T cells (Dieili *et al.*, 2003).

Figure 4: Structural organization of a granuloma.
*M. tuberculosis* reactive γδ+ T cells have been detected in the peripheral blood of tuberculin skin test (TST) positive and BCG-vaccinated individuals. These cells were found to be cytotoxic for monocytes pulsed with *Mycobacterial* antigens and to secrete cytokines that may be involved in the granuloma formation (Cooper, 1993; Munk *et al.*, 1990).

The role of γδ+ T cells in the granuloma formation in response to *M. tuberculosis* has been demonstrated in studies using mice with severe combined immunodeficiencies (SCID). In these studies, SCID mice did not form granulomas and rapidly succumbed to disease after BCG infection. However, these mice survived BCG inoculation, when engrafted with co-isogenic lymph node cells depleted of CD4+ and CD8+ T cells, indicating that the remaining γδ+ T cells were responsible for this response (Izzo & North, 1992; North and Izzo, 1993).

### 2.11.4 Macrophages

Apart from their significant function in innate immunity, macrophages have been reported to play a crucial role in the adaptive immune responses against *Mycobacteria* by producing cytokines such as tumor necrosis factor-alpha (TNF-α) and IL-1β (Fenton and Vermeulen, 1996). The importance of TNF-α has been extensively studied in knock-out mice. In these studies, TNF-α knockout mouse presented a profound susceptibility to aerogenic infection with *M. tuberculosis* characterized by a reduced macrophage differentiation and granuloma formation that resulted in *Mycobacterial* overgrowth and rapid animal death (Saunders and Cooper, 2000). Additionally, TNF-α and IL-1β
along with IFN-γ produced by T cells, stimulate production of nitric oxide in macrophages. The production of nitric oxide and related reactive nitrogen intermediates by macrophages is considered to be an effective host-defense mechanism against microbial intracellular pathogens like *Mycobacteria* (Chan *et al*., 1992; Denis, 1991). In the murine model of TB, nitric oxide plays an essential role in the killing of *M. tuberculosis* by mononuclear phagocytes. For example, in the mouse strain with a genetic disruption for inducible nitric oxide synthetase (iNOS), infection with *M. tuberculosis* is associated with a significantly higher risk of dissemination and mortality. Although more controversial in humans, there is a growing body of evidence that nitric oxide produced by TB-infected macrophages has anti-*Mycobacterial* effects against *M. tuberculosis*. The precise mechanism(s) by which nitric oxide and other reactive nitrogen species antagonize *M. tuberculosis* is not known, but may involve disruption of bacterial DNA, proteins, signaling, and/or induction of apoptosis of macrophages that harbor *Mycobacteria*.

2.11.4.1 B cells

While the role of T cells in the protection against *Mycobacterial* infections is well established, the role of B cells and antibodies is less understood. Studies conducted in mice lacking B cells have been controversial, making it difficult to define the role of these cells in anti-*Mycobacterial* immunity. In this regard, it has been reported that B cells play no role at all (Johnson *et al*., 1997). On the other hand, other studies have suggested a role for B cells as APCs and in granuloma formation (Vordermeier *et al*., 1996), or a role in the regulation of chemokines and/or adhesion molecules expression leading to recruitment of neutrophils, macrophages and CD8+ T cells during early *M. tuberculosis*
infection. (Bosio et al., 2000). Moreover, attempts at passive vaccination with antibodies in man and mice have also produced contradictory results, having reported no effect (Glatman-Freedman and Casadevall, 1998) or inhibition of bacilli dissemination (Pethe et al., 2001) and prolongation of survival in infected animals (Teitelbaum, 1998).

2.12 Virulence factor

*Mycobacterium* does not possess classical virulence factors like those which are the major causes of disease due to other pathogens, e.g., toxins produced by *Corynebacterium diphtheriae*, *Escherichia coli* 0157:H7 *Shigella dysenteriae* and *Vibrio cholerae* and other factors like capsules and fimbriae. However, a number of structural and physiological properties of the bacterium are beginning to be recognized for their contribution to bacterial virulence and The pathology of tuberculosis.
Figure 5: Flow chart showing the progression of *M. tuberculosis* bacillus after inhalation and entry into macrophages and activation of immune response.
2.13 Mechanisms for cell entry

The tubercle bacillus can bind directly to mannose receptors on macrophages via the cell wall associated mannosylated glycolipid, LAM, or indirectly via certain complement receptors or Fc receptors. On the other hand, surfactant protein D, similarly located in alveolae, inhibits phagocytosis of *M. tuberculosis* by blocking mannosyl oligosaccharide residues on the bacterial cell surface (Ferguson *et al.*, 1999), and it is proposed that this prevents *M. tuberculosis* interaction with mannose receptors on the macrophage cell surface. Cholesterol in cell plasma membranes is thought to be important for this process, since removal of this steroid from human neutrophils decreases the phagocytosis of *M. kansasii* (Piddington *et al.*, 2001) and similar depletion experiments prevented the entry of *M. bovis* BCG into mouse macrophages (Gatfield and Pieters, 2000). The human toll-like receptor 2 (TLR2) also plays a role in *M. tuberculosis* uptake (Frischkorn *et al.*, 1998).

*Intracellular Growth.* This is an effective means of evading the immune system. In particular, antibodies and complement are ineffective. Once M. TB is phagocytosed, it can inhibit phagosome-lysosome fusion. The exact mechanism used by M. TB to accomplish this is not known but it is thought to be the result of a protein secreted by bacterium that modifies the phagosome membrane. Ca$^{2+}$ signaling is inhibited when *M. tuberculosis* enters human macrophages but not when killed *M. tuberculosis* or antibody-opsonized *M. tuberculosis* cells are phagocytosed (Malik *et al.*, 2000). This effect was correlated with trafficking to late endosomes; i.e., elevated Ca$^{2+}$ levels were associated with phagolysosome Ca$^{2+}$ levels would help *M. tuberculosis* avoid
these host defense mechanisms. It has also been postulated that a selective advantage to *M. tuberculosis* of staying in an early endosome is that there would be less host immunosurveillance by CD4+ T cells. In agreement with this idea, there is a decrease in the expression of major histocompatibility complex class II (MHC-I) proteins and in the MHC-II presentation of bacterial antigens in macrophages after *M. tuberculosis* infection (Noss et al., 2001). This effect seems to be induced by presence of the secreted or surface-exposed *M. tuberculosis* 19-kDa lipoprotein, which is thought to interact with TLR-2 in the early phase of bacterial entry into macrophages (Thoma-Uszynski et al., 2001). The mechanism by which virulent *Mycobacteria* prevent phagosomal maturation is not known, but in the normal maturation of the *Mycobacterial* phagosome there is a successive recruitment of Rab proteins, which are small GTPases involved in endosome trafficking; i.e., Rab5 associates with early endosomes, and Rab7 is found in later endosomes. The *M. tuberculosis* phagosome that does contain Rab5 does not recruit Rab7 (Via et al., 1997). Also, TACO, a member of the coronin family of actin binding proteins, is preferentially recruited to the *Mycobacterial* phagosome of infected murine macrophages, where it was reported to be retained in phagosomes containing live and not killed *M. bovis* BCG (Ferrari et al., 1999). The bacterium may remain in the phagosome or escape from the phagosome, in either case finding a protected environment for growth in the macrophage.

*M. TB* interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by two mechanisms:
1. Compounds including glycolipids, sulfatides and LAM down regulate the oxidative cytotoxic mechanism.

2. Macrophage uptake via complement receptors may by-pass the activation of a respiratory burst.

Antigen 85 complex: This complex is composed of a group of proteins secreted by M. TB that is known to bind fibronectin. These proteins may aid in walling off the bacteria from the immune system and may facilitate tubercle formation although evidence of this is lacking.

Slow generation time. Because of M. TB’s slow generation time, the immune system may not readily recognize the bacteria or may not be triggered sufficiently to eliminate them. Many other chronic disease are caused by bacteria with slow generation times, for example, slow-growing M. leprae causes leprosy, Treponema pallidum causes syphilis, and Borrelia burgdorferi causes Lyme disease.

High lipid concentration in cell wall, as mentioned previously, accounts for impermeability and resistance to antimicrobial agents, resistance to killing by acidic and alkaline compounds in both the intracellular and extracellular environment, and resistance to osmotic lysis via complement deposition and attack by lysozyme.

Cord factor: The cord factor is primarily associated with virulent strains of M. TB. It is known to be toxic to mammalian cells and to be an inhibitor of PMN migration. However, its exact role in M. TB virulence is unclear.
2.14 Anti-Mycobacterial agents

Drugs used to treat tuberculosis are classified as first-line and second-line agents. First-line essential tuberculous agents are the most effective and are a necessary component of any short courses therapeutical regimens. The three drugs in this categories are a fat soluble complex, mycroyclic antibiotic Rifampin (RIF), water soluble hydrazides of isonicotin acid, Isoniazid (INH), and a derivative of nicotinic acid, Pyrazinamide (PZA). The first-line supplemental agents, which are a highly effective and infrequently toxic, include a water-soluble derivative of Ethylenediamine ethambutol (EMB) and an aminoglycoside isolated from Streptomycine griseus, Streptomycine (SM). Second-line anti-tuberculous drugs are much less effective than the first-line agents and much more frequently elicit severe reactions. They include a complex cyclic polypeptide derived from streptomycyes capriolus, Capriomycin, aminoglycosides like amikacin and kanamycin, para-amino-salisylic acid (PAS), a type of isoniazid, thiacetzone a complex based polypeptide antibiotic, viomycin, a derivative of isonicotinic acid, ethionamide and amino derivative of isoxazolidinone, cycloserine. Newer anti-tuberculous drugs, which have not yet been placed in first-line essential anti-tuberculous agents, the first-line supplemental agents and second-line antituberculous drugs include a semi-synthetic Rifamycin supiropiperidly derivative, Rifabutin, a semisynthetic cyclopropyl rifamycin antibiotics, rifapentine and fluorinated quenolonese like Ofloxacin, Ciprofloxacin, Sparfloxacin and Pefloxacin. Because administration of a single drug often leads to the development of a bacterial population resistant to that drug, effective regimens for the treatment of TB must contain multiple drugs to which the organisms are susceptible.
When two or more drugs are used simultaneously, each helps prevent the emergence of tubercle bacilli resistant to the others. However, when *in vitro* susceptibility of a patient's isolate is not known, which is generally the case at the beginning of therapy, selecting two agents to which the patient's isolate is likely to be susceptible can be difficult, and improper selection of drugs may subsequently result in the development of additional drug-resistant organisms.

Hence, tuberculosis is usually treated with four different antimicrobial agents. The course of drug therapy usually lasts from 6-9 months. The most commonly used drugs are rifampin (RIF) isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) or streptomycin (SM). When adherence with the regimen is assured, this four-drug regimen is highly effective. Based on the prevalence and characteristics of drug-resistant organisms, at least 95% of patients will receive an adequate regimen (at least two drugs to which their organisms are susceptible) if this four-drug regimen is used at the beginning of therapy. Furthermore, a patient who is treated with the four-drug regimen, but who defaults therapy, is more likely to be cured and not relapse when compared with a patient treated for the same length of time with a three-drug regimen.

### 2.15 Cell Wall

The shape forming properties of the bacterial cell walls are attributing to the peptidoglycan, whose chemical structure in *M. tuberculosis* closely resembles that found in other bacteria. Attached to this by phosphodiester bond is a branched chain polysaccharide, the arabinogalctan, whose distal ends are
esterified with high-molecular weight fatty acid (mycolic acids) of sizes and structure unique to *Mycobacteria*. Mycolic acids are 1-alkyl branched 2-hydroxy fatty acids, typically with 70 to 90 carbon atoms. The branch is commonly about 24-carbon atom long and is a simple alkyl chain, but the main chain contains (*M. tuberculosis*) cyclopropyl, Methoxyl or keto and methyl groups. This asymmetry between the two-alkyl chains is important in the construction of models of *Mycobacterial* envelope. Peptidoglycan-arabinogalctan Mycolate forms the so-called cell wall skeleton, which is readily isolated and studies associated with the skeleton, which is a large variety of lipids and glycolipids (and possibly some proteins). These difference considerable among *Mycobacterial* species or group of species and a few have highly distinctive ultra structural appearances, but those associated with *M. tuberculosis* (many of which are well studied and well understood from a chemical point of view) are not among the ultra structurally recognizable types. None of these associated molecules is the anatomical situation in *M. tuberculosis* known and their connections with ultra structure are not definitely known. New wall-associated molecules continue to be described and it seems certain that some remain to be isolated and identified. Consequently, one must admit the possibility that some features of the appearance of the wall are caused by substances still unknown.

The special ability of *M. tuberculosis* to survive in a mammalian host and to cause a potentially fatal disease presumably derives at least in part from the nature and arrangement of particular chemicals in the bacterial cell. Further
study of the ultrastructure of this particular species is needed to understand its pathogenic properties.

2.15.1: Cell envelope

The *Mycobacteria* cell envelope contains high proportion lipids, whose diverse structures have obvious chemo systematic potential. It is lipid part of cell wall that gives *Mycobacteria* their unusually thick and waxy envelop. The elaborate and distinctive feature of many of the cell wall moieties has led to speculate that these are involved in the virulence and pathogenesis of *Mycobacterium*. These structure include mAGP, LAM, LM, PIM, SL trehalose, 6,6'-dimycolate (cord factors), other acylated trehalose, phenolic glycolipid, lipooligosaccharide and attenuation indicator lipid. While some of these molecules, such as SL and cord factor, have previously been implicated in host-pathogen interaction (Goren and Brennan, 1979).

Peptidoglycan forms the basal layer of cell wall to which arabinogalctan and in turn, Mycolate are covalently attached. The peptidoglycan in *Mycobacteria* is of a common in many bacteria (Draper, 1982; Dean *et al.*, 2001) but with two slight differences. First, there are interpeptide linkages between two diaminopimelate residues as well as the usual D-alanyl-diaminopimelate linkages (Wietzerbin *et al.*, 1975). Second, the usual N-acetyl-muramic acid is replaced with N-glycolyl-muramic acid *M. bovis* and in other *Mycobacteria* (Azuma *et al.*, 1970).
Figure 6: Complex cell wall structure of Mycobacteria.
2.16: Clinical identification and prevention

Diagnosis of tuberculosis is a high index of suspicion. Diagnosis is not difficult with a high-risk patient. On the other hand it can easily be missed in a patient with a focal infiltrate. Diagnosis of tuberculosis requires detection of acid-fast bacilli in appropriate specimens depending upon the type of tuberculosis. Sputum is the most common specimen submitted for culture when pulmonary tuberculosis is suspected. Although not as satisfactory as expectorated sputum, induced sputum is a better specimen than gastric aspirates for the recovery of *Mycobacteria*. Bronchoscopy specimens or transtracheal aspirations are also appropriate respiratory tract specimens for *Mycobacterial* culture. In case of extra pulmonary tuberculosis appropriate specimens include blood, pleural fluid, bronchial, aspirates, bone marrow, purulent exudates, joint fluid, cerebrospinal fluid, nasopharyngeal swab and others. The examination of feces is usually not of value, since cases of intestinal tuberculosis are extremely rare. However, in patients with the acquired immune deficiency syndrome (AIDS) acid-fast stain and cultures of feces may be necessary for the efficient detection of *Mycobacteria* (Hart and Sutherland, 1977). M. TB can be cultured from specimens for definitive diagnosis. *Mycobacteria* have an extended generation time (20 - 22 hours) when compared with those of common bacterial species (40 – 60) which may appear in respiratory tract specimens. Therefore, *Mycobacteria* may be rapidly over grown by other contaminating bacteria if the latter are not inhibited. Also, bacteria waste products may accumulate in the medium and potentially inhibit the growth of *Mycobacteria*.
The high lipid content of *Mycobacterial* cell walls provides more resistance to
strong acids and alkalis than is found with other bacteria. This resistance has
promoted the development of decontamination procedures used to eliminate
most common bacterial flora while maintaining the viability of *Mycobacteria*.

A number of alkaline digestion, decontamination solutions are commonly used
for eliminating bacteria from contaminated specimens. Sample treatment with
NaOH kills other contaminating bacteria but does not kill the M. TB present
because M. TB is resistant to alkaline compounds by virtue of its lipids layer.

2.16.1: Growth Requirement

*Mycobacteria* are among the least fastidious of the pathogenic
microorganisms. Growth requirements on artificial media include potassium,
magnesium, phosphorus and sulfur. Nitrogen requirements are supplied by
ammonium salts or whole egg ingredients and carbon requirements are
supplied by glucose or glycerol. *Mycopacteria* grow best in a pH range of 6.5
to 7.0. The bacteria is strict aerobe and require proper aeration for the
elaboration of certain enzymes and pigments. A CO$_2$ concentration from 5%
to 10% is necessary for the primary recovery of *Mycobacterium tuberculosis*
and other *Mycobacteria*. Carbon dioxide is slowly produced during growth of
the culture; however, a CO$_2$ atmosphere is required for the first 3 to 4 weeks
of incubation. Optimum culture conditions for *M. tuberculosis* also include a
relatively high humidity and an incubation temperature of 35° to 37° C.

Four different types of traditional culture media used for the recovery of
*Mycobacteria* are shown below and include egg-based, agar-based, liquid and
selective media. Egg-based media include the following ingredients: whole eggs, potato flour, salts, glycerol and varying concentration of malachite green, an inhibitory dye. This medium type does not contain agar but is solidified by heating to 85° to 90°C for 30 to 45 minutes (inspissations).

The selection of media for the recovery of *Mycobacteria* is important. Most laboratories chose a combination of traditional egg-based, agar-based and/or selective media for the optimal recovery of *Mycobacteria*. Although many combinations exist, an optimal one would include L-J, 7H9 and S7H11 medium, the BACTEC-460, Middlebrook 7H12 and Souton's medium. Because most species of *Mycobacteria*, including *M. tuberculosis*, grow slowly, 4 to 8 weeks may be required before growth is detected. Although *M. tuberculosis* may be presumptively identified on the basis of growth time and colony pigmentation and morphology, a variety of biochemical tests have traditionally been used for identification of *Mycobacterial* isolates. In today's laboratories, the use of liquid media with radiometric growth detection (e. g. BACTEC-460) and the identification of isolates by nucleic acid probes or high-pressure liquid chromatography of mycolic acids have replaced the traditional methods of isolation on solid media and identification by biochemical tests. These new methods have decreased the time required for isolation and speciation upto 2 to 3 weeks other systems for culture on liquid media non-radiometric detection have become available.

The commonly used automated technique, the BACTEC system developed by Johnston Laboratories Cockeysville, MD for rapid detection of *Mycobacterial*
growth in clinical specimens used liquid Middle Brook 7H12 medium containing $^{14}$C labeled palmetic acid for the radiometric detection of *Mycobacterial* growth. The $^{14}$C label is evolved as $^{14}$CO$_2$ during growth and respiration. When the *Mycobacterial* growth reaches a predetermined growth index of 10, as determined by the amount of $^{14}$CO$_2$ evolved; a positive result is recorded by the BACTEC system. M.TB growth can be detected in 9-16 days vs 4 - 6 weeks using conventional media. The presumptive diagnosis is commonly based on diagnosis of *Mycobacteria* based on finding of AFB (Acid-fast bacillus) on microscopic examination of diagnostic specimens. The presence of AFB does not provide information concerning the identification or viability of the organism, since all species of *Mycobacteria* are acid-fast. (Textbook of Bacteriology. 2005; Minnikin et al., 1984). Most modern laboratories are processing large numbers of diagnostic specimens using two acid-fast control-fuchsion methods, including the Ziehl-Neelson (Z-N) and Kinyoun procedures and a fluorochrome method using auramine o or auramine- rhodamin dyes.

2.16.2: Diagnosis

Purified Protein Derivative (PPD) skin testing: PPD is most widely used as screening for *M. tuberculosis* infection and it is performed as the tuberculin or Mantoux test. Skin Testing is performed as the tuberculin or Mantoux test. PPD is employed as the test antigen in the Mantoux test. PPD is generated by boiling a culture of M. TB, specifically old Tuberculin (OT). 5 TU (tuberculin units), which equals 0.0001 mg of PPD in a 0.1 ml volume is subcutaneously injected in the forearm. The test is read within 48-72 hours. The test is
considered positive if the diameter of the resulting lesion is 10 mm or greater. The lesion is characterized by erythema (redness) and swelling and induration (raised and hard). 90% of people that have a lesion of 10 mm or greater are currently infected with M. TB or have been previously exposed to M. TB 100% of people that have a lesion of 15 mm or greater are currently infected with M. TB. or have been previously exposed to M. TB (Kenneth Todar, Bacteriology. 2005). The test is of limited value in the diagnosis of active tuberculosis because of its low sensitive and specificity.

Sensitization with nontuberculous bacteria leads to false-positive results and the test is difficult to interpret. A positive tuberculin test may be due to active tuberculosis, past infection, BCG vaccination or sensitization by environmental Mycobacteria. Therefore, this test is more helpful in places where BCG vaccination is no longer used effectively (Garg et al. 2003).

False negatives are more rare than false positives but are especially common in AIDS patients as they have an impaired CMI response. Other conditions such as malnutrition, steroids, etc., can rarely result in a false negative reaction. Recently, a recombinant antigen (DPPD) encoded by a gene unique to the M. tuberculosis complex organisms proves to be better than PPD in the Mantoux test (Campos-Neto et al., 2001). It can facilitate a more specific diagnosis of tuberculosis since the DPPD gene is not present in nontuberculous bacilli.

A number of nontraditional diagnostic tests have been evaluated as adjuncts to above mentioned traditional standard laboratory diagnosis. The
most thoroughly investigated is based on a number of *Mycobacterial* proteinaceous (ESAT-6, 14kDa, MPT63, 19kDa, MPT64, and 38kDa) and non-proteinaceous antigens (Trehalose based glycolipids like cord factor, trehalose-6, 6' dimycolate, acetyltrehaloses and phenolglycolipids) and detection of antibody *Mycobacterial* antigens in specimen of involved sites (e.g. sputum for pulmonary, CSF for tuberculous meningitis, pleural fluid and biopsy samples for pleural disease) and also in bone marrow and liver biopsy, (Daleine, 1995; Garg *et al.*, 2003). This serological diagnosis has generally not been sufficiently sensitive to be clinically useful. It has been difficult to develop an ELISA utilizing a suitable antigen because *M. tuberculosis* shares a large number of antigenic proteins with other microorganisms that may not be pathogenic. Nonspecific tests, such as the measurement of adenine deaminase in pleural fluid, have been evaluated but have not gained acceptance.

Recently, the γ-interferon assay was assessed as a potential candidate to replace the Mantoux skin test. The assay was evaluated in groups of immigrants, health-care workers and *M. tuberculosis* and *M. avium* complex MAC patients. The efficacy of the Mantoux test in cases effective tuberculosis, and it detected three of the seven cases of MAC colonization (Bellete *et al.*, 2002). New generation of diagnostic test that are based on molecular biology techniques are helping to rectify existing flaws in tuberculosis diagnosis but tend to be too costly for use in low-income setting. The approaches based on molecular biology for tuberculosis diagnosis includes branched DNA, Signal amplification (Varelidziz, 1994), Strand displacement amplification (SDA)
(Wolinsky and Schaefer, 1973; Walker et al., 1992), Polymarese chain reaction (PCR) (Pottumarthy et al., 2000; Mileler et al., 1994; Altamirano et al., 1992; Eisenach et al., 1990; Verbon et al., 1990; Hance et al., 1989), Ligas chain reaction (LCR) (Jacob et al., 1993; De Wet et al., 1987), DNA amplification, Transcription mediated amplification (TMA) (Jonas et al., 1993; Gladwin et al., 1998), and utilization of constructed Reporter Mycobacterium phage (Jacob et al., 1993; De Wet et al., 1987).

2.16.3: Prevention

BCG vaccine

In 1908, Camille Guérin and Albert Calmette initiated their attempts to produce an anti-TB vaccine from a virulent bovine strain. In 1921, vaccination with BCG, an attenuated vaccine, was introduced (Sakula, 1983). The efficiency of the BCG vaccine has been questioned since its early use and therefore, a large number of trials have been carried out to determine its efficacy. In these studies it was found that, the BCG vaccine protected efficiently against leprosy (Fine and Rodrigues, 1990) as well as childhood manifestations of TB (disseminated TB) (Rodrigues et al., 1993). However, the protective efficacy against pulmonary TB was limited (Tuberculosis Research Centre (ICMR), Chennai, 1999).

Many hypotheses have been suggested to explain the low protective efficacy of BCG against pulmonary TB. These hypotheses include inappropriate treatment and storage of the vaccine, the use of different strains of BCG (Fine, 1995), and lack of an effective stimulation of the optimal blend of T cell populations and in particular that of the CD8\(^+\) T cells (Hess and Kaufmann,
1999). In addition to these hypotheses, the currently used intradermal route of immunization has been suggested as another factor influencing the capacity of BCG to induce optimal immunity in the lungs. In this regard, (i. n.) route of immunization has recently been evaluated as a possible route for BCG delivery, in mouse experimental models. Results from this study showed a high degree of protection against challenge with *M. tuberculosis* in BALB/c mice, following BCG vaccination (Falero-Diaz *et al.*, 2000). In a similar model, vaccination with BCG conferred as good, if not better protection than subcutaneous (s. c.) route, against challenge with virulent *M. bovis* (Lyadova *et al.*, 2001).

2.17.3: Prospects for new vaccines

Given the limitations of BCG in protection against adult pulmonary TB, there is a considerable scope for improved vaccination strategies. Immunological research has a key position in understanding the pathogenesis of TB, and thereby in developing novel designs for effective prophylactic vaccination, immunodiagnostic tools and immunotherapeutic agents. Two approaches have been considered for vaccine development. One involves the replacement of BCG by a more potent vaccination inducing immune responses capable of either complete elimination of the bacilli, or of reliable containment of persistent infection.

The second approach involves the post-exposure vaccination to boost immunity in individuals whose natural immunity has already been primed by infection or BCG vaccination (Young and Stewart, 2002). Indeed, over the past decade research efforts have been directed to evaluate potential vaccine
candidates as well as alternative routes of vaccine delivery, such as the i. n. route, in order to improve protection.

2.16.4: New vaccine candidates

A wide range of potential vaccine candidates have been generated and subjected to tests for protective efficacy in experimental model of infection. New vaccine candidates include live attenuated vaccines, subunit vaccines and DNA vaccines.

2.16.5: Live attenuated vaccines

Advances in the techniques required to genetically modify *Mycobacteria*, as well as the increase in the knowledge of the pathogenesis of the microorganism, have made possible to delete genes encoding for potential virulence factors in *M. tuberculosis*, thereby enabling the generation of attenuated mutants. In addition to attenuated strains of *M. tuberculosis*, natural attenuated *Mycobacteria*, such as *M. vaccae* and *M. microti*, are being studied as possible vaccine candidates (Nor and Musa, 2004). Another approach has been the improvement of the BCG immunogenicity by the addition of genes encoding cytokines, such as IFN-γ (Murray *et al.*, 1996) or *Mycobacterial* proteins, such as the antigen 85 complex (Ag85) (Horwitz *et al.*, 2000).

Although encouraging results have been obtained in challenge experiments (Horwitz *et al.*, 2000; Smith *et al.*, 2001), a major consideration for the clinical use of live vaccines is safety, specificity when considering TB vaccination strategies for AIDS patients.
2.16.5.1: Subunit vaccines

Subunit vaccines are currently the most widely studied. This type of vaccine has been focused in particular on proteins present in filtrates prepared from \textit{in vitro} cultures of \textit{M. tuberculosis}, although non-secreted antigens have also been shown to induce protective responses in experimental studies (Coler \textit{et al.}, 2001; Skeiky \textit{et al.}, 2000).

The most extensively studied antigens are members of the Ag85 complex, a family of mycolyl transferases enzymes involved in cell wall biosynthesis and present in culture filtrates (Belisle \textit{et al.}, 1997). The Ag85 has been reported to induce strong activation of T cells in several studies (Andersen \textit{et al.}, 1995; Mustafa \textit{et al.}, 1998). Other antigens being studied are:

(i) Early secreted antigenic target (ESAT-6), which has been reported to be absent from all BCG vaccine strains and to induce very strong T cell and antibody responses (Brodin \textit{et al.}, 2004).

(ii) Heat-shock proteins (HSP), such as HSP-65 and HSP-70, found to induce a prominent immune response at both, the antibody and the T cell levels (Silva, 1999).

(iii) PstS-1 (38 kDa protein), a glycoprotein exposed on the surface of the bacillus and reported to be a powerful B and T cell antigen (Bothamley \textit{et al.}, 1992; Lefevre \textit{et al.}, 1997).

(iv) 19 kDa protein, a lipoprotein found to induce the expression of IL-12 and iNOS in monocytes and dendritic cells through its binding to TLR2
(Brightbill et al., 1999; Thoma-Uszynski et al., 2000) and to promote neutrophil activation (Neufert et al., 2001).

A limiting factor of the subunit vaccines is the need of adjuvant for vaccine delivery. Currently research studies are focused on the choice of which adjuvant to use and whether immunomodulatory, such as cytokines, should be used. Despite this drawback, subunit vaccines based on recombinant protein antigens are attractive because the techniques for production are established and this type of vaccine is expected to satisfy the regulatory requirements for use in humans more easily than the live vaccines.

2.16.5.2: DNA vaccines

Administration of naked DNA has the potential of eliciting both, cellular and humoral immunity against encoded antigens. Several Mycobacterial antigens, including the PstS-1, HSP-65 and the Ag85 have been studied and found to induce protection in animal models (Bonato et al., 1998; Fonseca et al., 2001; Huygen et al., 1996). Although the results are promising, concerns about the safety of DNA vaccination have been raised, mainly regarding the possibility of DNA integration into the host genome affecting oncogenes or tumor suppressor genes and thereby inducing the development of cancer. However, the risk of integration has been reported to be low under a variety of experimental conditions (Manam et al., 2000; Martin et al., 1999).

2.16.5: Experimental animal models in TB

Discussions about the value of experimental animal models in TB research have a longstanding history. Experimental animal models are critical for
delineating the general mechanisms underlying natural resistance, and acquisition of a protective immune response against TB. However, assessment of this information using experimental animals should be conducted carefully since there are differences in the host defense mechanisms between experimental animals and humans.

Many experimental animal species such as mouse, guinea pig, and non-human primates, have been used for deciphering the mechanisms involved in TB. The mouse, without doubt, is a very sophisticated and cost-efficient model. The immune response of the mouse is very well understood, and reagents such as monoclonal antibodies against surface antigens and cytokines are available. More importantly, the genetic manipulation of this species is highly advanced. Trans gene expression, gene knockout, gene knock-in have all become standard technologies, and a large variety of mouse mutants with defined immunodeficiencies are available to researchers studying the role of distinct cells and effector molecules in the _in vivo_ setting of TB. Moreover, the recent elucidation of the murine genome promises to open a new area of research with enormous impact on our understanding of genetic disorders and also of host mechanisms in TB (Kaufmann, 2003).

It is now well evident that tuberculosis poses a significant health rate threat to mankind. Multidrug resistant strains are on the rise, and _Mycobacterium tuberculosis_ infection is often associated with human immunodeficiency virus infection. Satisfactory control of tuberculosis can only be achieved using a highly efficacious vaccine.
Currently, two main vaccination strategies are being pursued. The first strategy uses subunit vaccines in the form of protein-adjuvant formulation, naked DNA, or recombinant bacterial or viral carriers that express defined antigens. Promising results have been obtained, but so far no vaccine candidate tested in animal models has proven to be better than BCG. The second strategy, comprising viable Mycobacterial vaccines, either attenuated viable M. tuberculosis or BCG, or recombinant BCG over expressing certain antigens or immunomodulatory, is also being pursued and shows promise. Recently the attentions have been focused on secretory protein antigens of Mycobacterium, which are synthesized by the actively growing M. tuberculosis culture and to induce the desired immune response (Anderson and Heron, 1993; Anderson, 1994). These proteins have also been termed as culture proteins and known to elicit strong immune reaction in humans and animals infected with M. tuberculosis/ M. bovis (Anderson et al., 1991 b; Orme et al., 1992; Romain et al., 1993; Anderson, 1994).

As a result of the combined efforts of several laboratories, more than 30 secretory proteins of M. tuberculosis have been characterized, (Andersen et al., 1991; Anderson, 1994, Kamath et al., 1999; Sonnenberg and Belisle, 1997; Karin et al., 1998; Gennaro, 2000; Kanaujia et al., 2004; Spencer et al., 2004; Young et al., 2004; Sable et al., 2005). These proteins are known to elicit strong immune reactions in humans infected with M. tuberculosis / M. bovis (Anderson et al., 1991; Orme et al., 1992; Romain et al., 1993; Anderson 1994; Ingrid et al., 2000; Sable et al., 2005).
The secretory proteins have been demonstrated to be strongly recognized by T-cells isolated from human (Tuberculosis) TB patients (Orme, 1996; Spencer et al., 2004) as well as mice and cattle experimentally infected with tuberculosis (Anderson and Heron, 1993; Pollock and Anderson, 1997; Lanbo et al., 2004). Experimental work in animal models suggests that both CD_{4}^{+} and CD_{4}^{+} T cells are required for optimal protection against tubercle bacillus (Orme et al., 1992; Bonato et al., 1998; Flynn et al., 1992; Pais et al., 1998; Spencer et al., 2004).

These proteins have been the focus of much of the research directed at identifying antigens that induce protective immunity or those that elicit immune responses of diagnostic value (Aub et al., 2000; Lein et al., 1999; Young et al., 2004; Paolo et al., 2004).

The recent identification of novel secreted proteins of *M. tuberculosis* open the way to studies on their immunological characterization of these protein to define their potential for immunological diagnosis of tuberculosis or vaccine design.

A few numbers of secretory antigenic protein and peptides from M. TB have already been evaluated as antigens for the immunodiagnosis of tuberculosis. In an effort to develop more accurate diagnostic tools, and suitable vaccine the present research program is aimed to assess the diagnostic and preventive potential of certain secretory candidate proteins.
The *M. tuberculosis* culture filtrate, which contains as many as 200 proteins (Romain *et al.*, 1993; Ingrid *et al.*, 2000), has been investigated by means of protein purification by immunological methods, and by screening of expression libraries of *M. tuberculosis* DNA with anti-culture filtrate sera (Wolinsky and Schaeferm, 1973; Ginsberg, 1998; Grange and Laszlo, 1990; Bothemley *et al.*, 1991; Altamirano *et al.*, 1992; Sorensen *et al.*, 1995; Mileler *et al.*, 1994; Bellete *et al.*, 2002).

The early secretory antigenic target (ESAT)-6, purified protein and peptides from *Mycobacterium tuberculosis* have been already under evaluation as antigens for the immunodiagnosis of tuberculosis (TB). Some important antigens, the (38 KDa, 30/31 KDa, 40 KDa, 42 KDa, SOD, 30 KDa MSP, 85B, ESAT-6, and CFP10) molecules, have been found to be secreted by *Mycobacterium tuberculosis* (Roberts *et al.*, 1995: Ulrichs *et al.*, 1998; Gennaro, 2000; Smith, 2003: Spencer *et al.*, 2004). These *Mycobacterial* antigens are already under process in various laboratories to use as markers for tuberculosis diagnosis by using ELISA and other non-traditional diagnostic technology.

As we are aware of effect that one of the main objectives of the research in the field of *Mycobacteriology* is the development of new methods that will improve and expedite the diagnosis and treatment of tuberculosis and other *Mycobacterial* infections. Some forms of tuberculosis are difficult to diagnose by the available routine diagnostic methods. In spite of new technologies, no reliable new serological test has been developed for the diagnosis of
tuberculosis. No reports are available on the diagnostic and protective efficacy of low molecular weight M. TB secretory proteins for patients suffering with TB. Therefore present research has been investigated for identification of low molecular weight *Mycobacterial* antigenic secretory proteins and development of immunodiagnostic tool for detection and diagnosis of tuberculosis.

The study has been focused towards the following properties with special attention- High sensitivity, High specificity, Reproducibility and Stability.

This will be simplify the effort towards the development of more efficient immunodiagnostic system, effective vaccine and immunotherapy for the prevention and therapy of tuberculosis although diagnostic system based on diverse native epitopes are developed but a continuous effort is required to further evolve more and more efficient diagnostic tools.

Research design to apply isolated and screened low molecular weight secretory proteins with special reference to immunoprophylactic activity and development of a novel diagnostic tool, by using searched antigenic low molecular weight secretory protein to detect anti-*Mycobacterial* antibodies by various possible designed tools in the form of Tri Dot/ ELISA/ Bi Spot/ Immuno-chromatography, finally the developed diagnostic product will be evaluated to find out its applicability and acceptability under following: Sensitivity, Specificity, and Feasibility in the field and immuno protective for subunit vaccine.