General Discussion
Tuberculosis is a disease of global importance. One-third of the world’s population is estimated to be infected with *Mycobacterium tuberculosis* and eight million new cases of tuberculosis arise each year.

Since one third of the world's population carries *M. tuberculosis* in its latent form, and that 5% develop active disease during the first years of infection, it is estimated that 8 million new cases of tuberculosis and 3 million deaths occur each year (Dye, 1999 and WHO, 2004; Stefan and Andrew, 2005). Furthermore, the risk of reactivation increases as a consequence of associated pathologies, immunosuppressive therapy, malnutrition and, mainly coinfection with human immunodeficiency virus (HIV) (Rook GAW and Pando, 1996). Identification of these individuals is crucial for the control of disease transmission and elimination of tuberculosis.

The ability of a protein to detect antibodies present during subclinical disease is as important as the sensitivity of a protein in detecting antibodies formed during active tuberculosis, since therapy for latent tuberculosis infection can prevent the development of active disease.

Several antigens of *M. tuberculosis* have been found to be useful in the serodiagnosis of clinical disease (Bisen *et al.*, 2003; Garg *et al.*, 2003; Garg *et al.*, 2004), Traditional methods (smear and culture) are simpler and less expensive than the new molecular diagnostic tests such as PCR that are based on amplification of nucleic acid. Serological methods seem to be the ideal choice and, thus many *Mycobacterial* antigens like cellular extracts, proteins, (Arikan *et al.*, 1998) polysaccharides, DNA, RNA (Bisen *et al.*, 2003;
Garg et al., 2003; Garg et al., 2004) glycolipids (Julian et al., 2002, Papa et al., 1989) and other biomolecules have been evaluated. (Tiwari et al., 2005).

It is evident from several studies that resistance against tuberculosis is mediated by arm of the immune system. Therefore, the antigen for vaccine development should be selected on its ability to induce the cell mediated immune response. In past, attempts have been made to investigate the immunogenic potential of different components of Mycobacterium. Several components viz. Mycobacterial cell wall, Heat shock proteins, ribosome/RNA and mannosides have been evaluated for their immuno protective potential (Mehta, 1988; Hetzel et al., 1998; Oliver et al., 2000; Gennaro, 2000; Leander et al., 2005; Kwasi et al., 2005).

Recently, the attention has 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 (Andersen et al., 1991; Anderson, 1994; Kamath et al., 1999; Sonnenberg and Belisle, 1997; Karin et al., 1998; Kanaujia et al., 2004; Spencer et al., 2004; Young et al., 2004; Sable et al., 2005). These proteins have also been termed as culture filtrate proteins (CFP) and known to elicit strong immune reactions in humans and animals infected with M. tuberculosis / M. bovis (Anderson et al., 1991; Orme et al., 1992; Romain et al., 1993; Anderson, 1994).

The secretory proteins have been demonstrated to be strongly recognized by T-cells isolated from human (Tuberculosis) TB patients (Orme, 1988a) as well as mice and cattle experimentally infected with tuberculosis (Anderson and
Experimental work in animal models suggest that both CD_{4}^{+} and CD_{8}^{+} 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; Stefan and Andrew, 2005).

Secretary proteins, for comparison, are broadly recognized early during disease development in different species infected with *M. tuberculosis* (Anderson et al., 1995; Brandt et al., 1996; Pollock and Andersen, 1997; Kanaujia et al., 2003).

They have discriminated TB patients from both BCG vaccinated and *M. avium* patients and have therefore been suggested as candidates for in vitro TB diagnosis (Lein et al., 1999; Ulrichs et al., 1998).

A combination of secretory protein (CFP) antigens selected by our approach can provide clues, which would allow the host immune responses to be better understood. This approach had also been successful in bringing about selection and use of antigens, which may be utilized for serological based diagnostic assay for tuberculosis and subunit vaccine.

Our efforts were intended to focus on secretory protein antigens, which can be suitable for developing a reliable diagnostic test for tuberculosis and utilization of subunit vaccine (protective efficacy). Such antigens, if found, can be easily incorporated into Enzyme Linked Immunoassays or all those related assays which utilize antibody antigen interactions as a marker of diagnosis, as has been utilized by others (Hirayama et al., 2005).
Since the ELISA technique has the advantage of being a semi automated procedure with the capacity to analyze multiple samples and the equipment needed is available in many hospitals even in the developing countries of the world. As opposed to other infections, the use of ELISA in the diagnosis of tuberculosis has never been widely implemented due to problems with both sensitivity as well as specificity. For developing such assays, it was desirable to find out and purify Secretory protein (CFP.) antigens, which may specifically diagnose active tuberculosis sufferers.

The present study was investigated to examine the immuno diagnosis and immunoprotective activity of the secretory proteins of Mycobacterium tuberculosis H37Rv. The Secretory proteins were isolated from logarithmic phase culture (5 week old) of Mycobacterium tuberculosis H37Rv without shaking at 37°C (Nagai et al., 1991). It has been earlier demonstrated that the cultivation time, growth of bacteria can be examined by microscopy (Figure-9) and spectrophotometer at A580 (Figure- 11). Protein secretion was monitored through the protein estimation of culture medium by at A280 (Figure-12) and the profile of proteins released into the culture supernatants of the tubercle bacilli by SDS-PAGE.

The major secretory proteins were purified by following the method of Nagai et al. (1991). For initial fraction of major secretory proteins, DEAE sepharose CL-6b anion exchange column equilibrated with Tris-HCl was used. The five proteins (6 kDa, 26, 30,38 and 64 kDa) in peak I, II, III, IV, V, and VI were virtually obtained in pure form in this column and characterized by SDS PAGE
and Tricine SDS PAGE (low molecular weight proteins); again the individual protein band were then eluted from the gel. The reactivity of secretory protein antigens were confirmed by western blotting with positive tuberculosis patient’s sera and ELISA.

We selected for the study, secretory protein antigens with molecular weight of 6 kDa, 26 kDa, 30 kDa, 38 kDa and 64 kDa for serodiagnosis and protective efficacy.

We used Western blot because it is a method that provides a safe reading and interpretation of the results when duly standardized. In the present study, we produced industrial lots of antigen fractions that were assessed for reproducibility with control serum samples collected from patients with confirmed pulmonary tuberculosis and healthy individuals. Compared to molecular methods, Western blot has the advantage of being easy to perform, considering that small laboratories are better equipped for the execution of serological tests, detection immuno reactivity than of molecular tests. The stability of the nitrocellulose strips, that can be stored for more than 12 months, permits the execution of the test in laboratories with small routines. The Western blot method has been widely used to confirm serologic results and for the identification of the immune response against different protein fractions of viruses, fungi, and parasites (Jackett et al., 1988).

Studies employing Western blot analysis have shown that M. tuberculosis secretory protein antigens in the range of 64, 38, 30, 26 and 06 kDa frequently react with tuberculosis positive serum (Figure-18 to 23).
Further, we also used Enzyme-Linked Immunosorbent Assay (ELISA) because it is a powerful method in estimating ng/ml to pg/ml ordered materials in the solution, such as serum, urine, and culture supernatant. There are different kinds of ELISA employed for different conditions. In these studies, since the antigens/antigenic epitopes were developed and very well characterized, indirect ELISA was found most suitable for detecting their corresponding antibodies in the solutions.

1. In indirect ELISA, if solutions contain antibodies to the antigenic epitopes, those antibodies bind to the corresponding secretory protein antigens on the plate. Unbound antibodies are washed out and followed by reaction with anti-human immunoglobulin coupled to an enzyme (e.g., Horse, radish Peroxidase). This is the second antibody that binds to human antibodies captured on the plate. Unbound enzyme coupled immunoglobulins are washed out and are followed by reaction which further changes the color of chromogen. The enzymatic reaction is quenched after an optimum time period by denaturing the enzyme. The color of final product is measured calorimetrically and interpreted.

When considering the binding capacity of adsorbent plastic surfaces for biomacro-molecules, it is essential to distinguish between the total amount of molecules that can be bound to the surfaces and the amount that can be bound and still remain biologically active. Both quantities are very much dependent on the nature of the molecules and the character of the surface. The adsorption of molecules to a polystyrene surface is due to inter-molecular
attraction forces (van der Waals forces), to be distinguished from true chemical bonds, i.e. covalent bonds (through electron share) and ionic bonds (through stoichiometric charges of opposite signs). There are four main types of possible bonds between macromolecules.

a) True chemical bonds are represented by a covalent disulphide bond.

b) Ionic bond between a carboxyl ion and an amino ion.

c) Van der Waals mediated bonds are represented by a hydrogen bond between two dipoles and an alternating polarity bond between hydrocarbon residues protruding from the macromolecules backbones, Where the encircled area indicates a water-deprived zone.

There are two different types of absorbent polystyrene surfaces that are available from Nunc.

- POLYSORB surface
- MAXISORB surface

PolySorp Predominantly presents hydrophobic groups, MaxiSorp has in addition many hydrophilic groups, which results in a fine patchwork of hydrophobic and hydrophilic binding sites. In this study MaxiSorp surface was used for facilitation of the absorption of hydrophilic synthetic epitopes, because not only can this surface compete with the water molecules for binding the macromolecules by hydrogen bonds, but the molecules can also be captured from a much longer distance by the long-range hydrogen bond forces for establishment of both hydrogen bonds and eventually hydrophobic bonds. However, with MaxiSorp peptide epitopes binding events are more
likely to occur, which means that adequate incubation conditions are easier to establish.

As mentioned above, van der Waal mediated bonds are relatively weak, therefore, they may be insufficient for stable bonding when they are few in number, i.e. when the molecules are small. For binding of small molecules, strong chemical bonds are needed. Ionic bonds would not do, because they normally dissociate in aqueous solution, leaving covalent bonds as the only possibility for direct, stable binding of small molecules e.g. peptides. However, this difficulty may be overcome by using small molecules linked to (indifferent) carrier macromolecules as supported by this study.

Antigen coating efficiency is dependent on immobilization pH (Coating buffer), ionic strength, immobilization time (incubation period), concentration of antigens, and temperature of immobilization.

Antigens showed maximum absorption on solid phase at pH 7.2 whereas cocktail of antigens showed maximum adsorption at pH 9.2. Since there was no much difference in the adsorption between pH 9.2 and 7.2. Hence, the common pH was selected for the immobilization of cocktail of epitopes.

All the antigens showed maximum absorption on solid phase between 10 mM to 50 mM but reactivity fall down at 100 mM. The saturation of the adsorption of all the epitopes on solid phase took place within 18 hours.
Investigation of immobilization of antigens with the variation in the concentration revealed that there is a rise in the concentration of all the protein antigens from 2.5 ug/ well to 7.5 ug/ well and then after, there was no effect of higher antigen concentration on immobilization as evident by the experimental data.

Work on the immobilization time at 37°C showed that there was rise in the adsorption of the antigens on the surface up till 120 minutes followed by saturation. Although optimum adsorption took place in 120 minutes, but it was advisable to prolong the incubation period for another 120 minutes.

Blocking agents are essentially to be used in ELISA for blocking possible excess solid surface after coating with antigen to avoid unspecific immobilization of succeeding reactants. One reason for using a true blocking agent would be to substitute detergent for blocking. If detergent is present during incubation with secondary reactants, it might in some way interfere with the immunologic specificities or cause unspecific immobilization of the reactants (Esser, 1990). If detergent is present during wash after secondary reactants, possible weak immunologic affinities might be broken by the washing activity of the detergent. Another reason for using a blocking agent would be to stabilize the immobilized antigens of coated surfaces. Typical blocking agents would be an indifferent macromolecule, large enough to establish a stable attachment to the surface, yet small enough to find its way between antigens.
Bovine serum albumin (BSA), of MW 67,000 is commonly used as a blocking agent. Also the more heterogeneous casein is often used and may be more effective than BSA (Pratt and Roser, 1989; Vogt et al., 1987). In this study Casein digest, hydrolysate was found to be the best blocking agent amongst all the three blocking agents studied. Casein digest, hydrolysate was found better than BSA (Table-III, Figure- 24), which was further found better than normal rabbit serum.

Casein digest, hydrolysate stood to be the best blocking agent amongst all the three studies due to its following features:

1. Due to its heterogeneous nature (different masses of peptides) it blocks the inter-epitope space and unimmobilized space on the solid surfaces more firmly than BSA and normal rabbit serum.

2. It also has exerted lesser effect on the stearic hindrance in antigen-antibody reaction.

3. It also has lesser effect on the shielding epitopes.

In this regards 0.1% of casein digest, hydrolysate, in phosphate buffered, 100mM, pH 7.2 was found sufficient enough to serve the purpose of blocking.

Assay employing the cocktail of selected antigen was evaluated using the specific antibodies to the antigens to validate the immobilization of particular antigens epitope and found that all the epitopes subjected for coating were immobilized optically. This assay differentiated the reactive and non-reactive samples.
Amongst all the reagents in ELISA, anti IgG-HRP conjugate, solid phase immobilized antigens and the enzyme substrate-chromogen (TMB-H₂O₂) are very critical, hence an effort was made to stabilize them. In the conjugate, HRP is very prone to peroxidation that was protected by a reducing agent (TMB). Protein part of the conjugate was protected from deterioration by protein stabilizers (casein digest, hydrolysate), preservatives (thiol containing compounds), surfactant (Tween – 20) and antibacterial compounds.

To determine the antibody response to secretory protein (CFP) antigens in tuberculosis patients, (Imaz and Zerbini, 2000). We determined the specific response to the 30kDa and 6kDa antigens. A possible explanation for this phenomenon may be the intense stimulation of the immune response by antigens released by bacteria, and / or by the release of antibodies that were previously part of immune complexes.

The response to the 30 or 6kDa secretory protein antigens was reactive in TB patients than in contacts or healthy subjects. The presence of these antibodies in tuberculosis patients has been associated with a more favorable prognosis or with spontaneously cured tuberculosis. Thus, the presence of these antibodies in contacts may suggest a tuberculosis infection that is not clinically evident.

It has been investigated that the antibody response against antigens in the 6kDa secretory proteins is suitable for diagnostic purposes as well as protective efficacy. The antigen possesses various specific T-cell epitopes that induce a cellular response and lead to increased interferon gamma production in patients with sub clinical or active tuberculosis but not in
unexposed healthy individuals (Arend et al., 2001). This cell-mediated response has also been associated with increased risk of disease (Doherty et al., 2002). A recent study showed that the humoral response to ESAT-6 may be associated with inactive tuberculosis but not with active tuberculosis (Silva et al., 2003).

These reactivity profiles showed sensitivity similar to that observed by other investigators. However, in the general population, especially in developing countries, with a high prevalence of *M. tuberculosis* infection, there are a percentage of persons who do not present clinical signs and symptoms of tuberculosis and who must be identified and treated. For this purpose, a highly specific test is needed in order to detect suspected cases.

Earlier studies have suggested that the humoral response to *M. tuberculosis* antigens was increased in patients with an inadequate cell-mediated response to the same antigens (Bhanrnagar et al., 1977) and in patients that showed a strong humoral response after the infection led to the development of active tuberculosis (David et al., 1992). This evidence, together with a recent study that evaluated the humoral response to recombinant *M. tuberculosis* antigens, 30kDa and 6kDa showing association of the latter two antigens with risk factors for future active, but not current disease suggest the possibility of identifying the subset of persons with latent tuberculosis infection who may be at high risk to develop active disease.

Since significant geographical variation in antibody titer to some antigens exists, heterogeneous recognition of antigens by serum antibodies in tuberculosis can result from multiple factors (Lyashchenko et al., 1998).
The humoral response mainly against antigens of mass 30kDa, 6 kDa and cocktail seems to be important for the detection of latent tuberculosis.

**Immunoreactivity of Purified Secretory Proteins**

Total culture filtrate proteins (CFP) when administered as CFP-IFA complexes elicited both humoral and cellular immune response (Table- X and Figure-31) to all the five purified secretory proteins. CFP elicited significant antibody response to all the five antigens throughout the observation period (fourth week). The optimal response was obtained at third week (Table-X and Figure-31) for 6kDa, 38kDa, 64kDa and CFP secretory proteins. The induction of high levels of antibodies to the various secretory proteins of *Mycobacteria* has been reported earlier by few workers in the animal and humans infected with *M. tuberculosis* (Pessolani *et al.*, 1989; Huygen *et al.*, 1996; Lanbo *et al.*, 2004) and in animals immunized with the culture filtrate proteins of *Mycobacteria* (Daugelet *et al.*, 1992; Andersen, 1994).

Besides the humoral response, enhanced sensitization, implying the activation of T-lymphocyte by the CFP against all the five antigens was seen by *in vitro* T-lymphocyte proliferation assay (Table-XI and Figure- 32). The proliferation activity was observed with all the purified proteins in the lymphocytes obtained from immunized animals. However, the maximum activation T-lymphocytes was seen with 6kDa secretory antigen throughout the time period with peak activity in third week p. im. as compared to the other antigen . This observation has been in concurrence with the reports of other workers that the secretory proteins are (Orme and Collins, 1983; Pal and Horwitz, 1992;
Andersen et al., 1991b; Andersen, 1994) the major targets of T-lymphocytes. This also explains the superiority of live BCG vaccine over the killed vaccine in evoking the strong immune response and protection (Smith, 1985) possibly due to the secretory proteins, which may be largely responsible for its protective efficacy. Further, the observation that 30 and 6kDa secretory proteins being the most immunoreactive protein of the five secretory proteins is in agreement with the observation that the secretory proteins of immune T-lymphocytes (Andersen et al., 1991; Hubbard et al., 1992; Daugelat et al., 1992; Andersen, 1994). Detailed investigations of immune responses were carried out with 6kDa secretory protein since, it was the most immunoreactive and major secretory protein as compared to other purified secretory proteins of M. tuberculosis H₃₇Rv (Table-X, XI and Figure- 31,32).

**Immunogenicity of 6 kDa Secretory Protein Complexed with IFA**

Different concentrations of 6kDa antigen (75 ug, 37.5 ug and 18.75 ug) when administered as 6kDa IFA complexes in animals elicited cellular and humoral responses (Table-XII and Figure- 33). An enhanced antibody level was demonstrated in all the groups of mice immunized with different concentrations of 6kDa antigen throughout the time period attaining its peak level in 4th week (Table-XII and Figure- 33). Similarly, there was significant T-lymphocyte proliferation in 6kDa-IFA immunized animals with different concentration of antigen. The pronounced T-lymphocyte proliferation activity of 6kDa protein in animals immunized with total culture filtrate might be due to the presence of several proteins in culture filtrate which perhaps influence the immunoreactivity of 6kDa secretory protein. Furthermore, the immune
responses in present study (cellular and humoral) were found to be dose depended demonstration higher activation of immune cells in animals immunized with highest concentration of antigen (75µg). Similar observation has been reported by Andersen (1994) that the increase in the doses of immunogens from 10-250 µg was associated with the increase of more than 100ug resulted in the shift of immune response from Th1 towards Th2 type, thus emphasizing that the dose of antigen should be carefully standardized to obtain the desired immune response.

Furthermore, the immune response induced with 75µg concentration of the antigen was higher (Table-XIII and Figure- 34) than observed in BCG vaccinated mice. Our observations are in agreement with earlier reports that the immunization with pure protein or combination of proteins induces better immune responses compared to that induced by the whole organism (Pal and Horwitz, 1992; Andersen, 1994; Ingrid et al., 2000; Kanaujia et al., 2004; Stefan et al., 2005).

Protective immunity in tuberculosis and the diseases caused by intracellular pathogens are mediated by specifically activated T-lymphocytes (Orme and Collins 1983; Blander and Horwitz, 1993). It is believed that the CD4⁺ T-lymphocytes act as primary effector cell population which is responsible for the induction of acquired resistance against the disease (Muller et al., 1987; Orme, 1988a). The CD4⁺ T- lymphocytes can be further differentiated into two major subsets Th1 and Th2 on the basis of cytokine profile secreted by the activated CD4⁺ T- lymphocytes (Mossaman et al., 1986; Stefan et al., 2005).
These findings suggest that 6kDa secretory proteins induce the activation of Th1 type of T-lymphocytes.

Therefore, it is concluded that 6-kDa secretory protein of *M. tuberculosis* H37Rv complexes with IFA elicited strong cell mediated and humoral immune response, which was skewed towards Th1 type of T-lymphocyte activation.

**Protection Conferred by 6 kDa antigen complexed in IFA**

BCG is the most widely used live vaccine in the prophylaxis against tuberculosis, the efficacy of which varies between 0-80% (Baily, 1990; Fine, 1989). Hence, efforts are being made to identify the immunoprophylactic components of *Mycobacteria*. Amongst the various *Mycobacterial* components, secretory proteins have been proposed to be important antigens for the development of subunit vaccine. *Mycobacterial* secretory proteins released by actively growing cells are established that 6 kDa secretory protein of *M. tuberculosis* H37Rv induced strong humoral and cell mediated immune responses when complexed in IFA, the prophylactic significance of this protein was explored. In experimental infections for the evaluation of new prophylactic agents, responses are strongly influenced by the choice of animals, route of administration of antigen and size of challenge dose (Ribi et al., 1965; Newman and Powll, 1995). The choice of mouse as an experimental model is justified, as it is cost effective and relatively easy to handle. It also has the advantage of well established genetic and immunological markers which allow detailed analysis of immune reactions during immunization and infection (Harboe et al., 1986). Similarly, the
immunization route used to deliver the subunit vaccines can dramatically influences the type of immune response generated (Fadda et al., 1998). The choice of peripheral route for immunization using IFA by us in present study appears to be justified as the inoculation of oil treated components leads to graulomatous response in animals (Barclay et al., 1967), also the LD₅₀ of *M. tuberculosis* H₃₇Rv was chosen because massive i. v. challenge with virulent *Mycobacteria* leads to a rapid response which masks the protective efficacy of vaccine.

The immunization of animals with different concentrations of 6kDa secretory protein complexes in IFA resulted in significantly higher percent survival (Table-XIV, XV and Figure-35) against an intravenous challenge with LD₅₀ of *M. tuberculosis* H₃₇Rv as compared to unimmunized animals. Moreover, the protection induced was found to be dose depended which was maximum (100%) in mice immunized with 75µg concentration of the antigen, again emphasizing the effect of dose the immunogens used to mediate desired immune response and protection. Since the mechanism of immunoprotection has been observed to be T-cell mediated (Orme and Collin, 1983; Andersen, 1994; Spencer et al., 2004; Landbo et al., 2004; Sable et al., 2005) the dose dependent increase in the protective efficacy is explainable.

Further, the immunization with 6kDa antigen demonstrated a significant percent survival, which was better than that observed in BCG vaccinated mice (72.7%) (Table- XV, XVI and Figure -35) and 45% in control groups, challenged at the peak of immune response with LD₅₀ of *M. tuberculosis*
H₃⁷Rv. Increased survival rates of animals actually reflects the retardation in the rate of multiplication of *tubercle bacilli* (Younmans *et al.*, 1976). Therefore, viable counts in the infected organs were enumerated. Immunized mice demonstrated a significant decrease in bacterial load thirty days after the challenge (Table-VI) in lungs, liver and spleen as compared to controls. This further supports the observed higher percent survival and also satisfies the criterion for the observed higher percent of vaccine (s) against tuberculosis (Weigeshoua *et al.*, 1970). The decrease in CFU's recovered from liver and lung in 6kDa immunized was better than that obtained with BCG vaccinated mice. The induced protection by 6kDa antigen was also better than that obtained with BCG vaccine by other workers (Orme, 1988a; Andersen, 1994; Roberts *et al.*, 1995). These findings are further substantiated by the earlier reports (Hubbared *et al.*, 1992; Pal and Horwitz, 1992; Andersen, 1994; Howitz *et al.*, 1995) where in the protective effect of the secretory proteins has been demonstrated to be comparable to BCG vaccine. Interesting, Andersen (1994) had shown the maximum immune reactivity in the region of 6kDa in ST-CF immunized and BCG vaccinated mice the major immunoprotective antigen of *M. tuberculosis* H₃⁷Rv.

Since, during the initial stages of immunization with antigen in Incomplete Freund's adjuvant (IFA), the nonspecific inflammatory responses might have affected the degree of protection and immune response, the protection efficacy was determined after 1ˢᵗ and 4ᵗʰ weeks of immunization to ensure that the nonspecific response due to antigen and IFA are diminished. The 6kDa-IFA immunized mice challenged 7 and 30 Days post immunization, demonstrated a percent survival and at thirty days post challenge only 30% of
the control mice survived the challenge. On the other hand, the mice immunized with varying concentration of the antigen demonstrated no mortality till two weeks post challenge. However, the onset of mortality occurred during third week post challenge with 20% 30% and 40% mortality in animals immunized with 75µg, 37.5µg, 18.75µg of the antigen respectively and 20% mortality was also observed in animals vaccinated with BCG vaccine. In the fourth week an increased mortality (50% & 60%) was observed in the lower two doses of 6kDa immunized animals. Mortality was also observed in 75µg protein (25%) and BCG vaccinated (30%) animals.

At the end of 30 days observation, 75% survival was seen in animals immunized with 75µg concentration of 6kDa protein that was similar to survival (70%) afforded by BCG vaccine.

Thus indicating that protection imparted by 6kDa secretory antigen is mediated by specifically activated T-lymphocytes. The observed decline in protective immunity to virulent challenge of *M. tuberculosis* H37Rv might be due to the protective immunity and heightened state of memory (Sprent, 1993) however, as the protection afford by 6kDa-IFA immunization during 3rd week remained stable indicates the induction of memory T-lymphocytes. The maintenance of stable state of immunity by BCG vaccine throughout the observations period could be due to the continuous stimulus provided by the secreted proteins released by actively growing cells. Our observations are supported by the findings (Pal and Howitz, 1992; Andersen, 1994; Roberts et al., 1995; Horwitz et al., 2000) that culture filtrate proteins of Mycobacteria
confer protective immunity, equivalent to the BCG vaccine. Further, the protection imparted by culture filtrate proteins was shown to be long lasting and mediated by T-lymphocytes (Andersen et al., 1994).

Thus our results also support the emerging hypothesis that secretory proteins are the key immunoprotective molecules capable of generating a strong cell mediated immune response leading to an efficient acquired resistance against virulent *M. tuberculosis* H₃₇Rv and other intracellular pathogens.

**Immunoprotective Activity of 6kDa Secretory Protein Entrapped in Liposome**

Previous observation about 6kDa secretory protein (CFP) of *M. tuberculosis* H₃₇Rv complex with FIA could induce both cellular and humoral responses. Furthermore, immunization with 6kDa-FIA was able to confer significant short and long-term protection against tuberculosis. Further, studies were carried out to evaluate the immunoprotective behavior of 6kDa secretory protein using liposome as adjuvants.

Although, FIA is the most commonly used adjuvant for experimental studies, it is far from idea with respect to human use because of severe side effects associated with it (Freund, 1956; Gupta et al., 1993). Thus, an adjuvant, which does not cause any adverse reaction, is required for development of alternate subunit vaccine. Recently, the use of a large number of adjuvants (FIA, RIBI adjuvant, DDA, ISCOMS, Saponins microspheres i. e. PLG and Liposomes) have been reported to be potent inducers of cell mediated immunity has emphasized the decisive influence of adjuvant on vaccine
efficacy. Among the different adjuvant systems, liposomes have been viewed as an alternative to alum based (only adjuvant licensed for human use) adjuvant. In earlier studies, the phosphatidylcholine (PC) liposomes have been shown to be promising adjuvants for proteins antigens (Lawman et al., 1981; Pimn and Baldwin, 1984; Pancholi et al., 1989; Chugh and Khuller, 1993). Further, incorporation of charge in PC liposomes, method of preparation and addition of other adjuvants such as Lipid A, muramyl dipeptide, alums, has been reported to boost the adjuvanticity of liposomes (Fries et al., 1992; Gupta et al., 1993).

In this study, immunogenic role of 6kDa secretory protein entrapped in liposomes has been investigated. Liposomes used in the study were prepared by Freeze-thaw method (Mayer et al., 1986) containing phosphatidylglycerol (PG), cholesterol (Ch) and adsorbed on to alums. Percent entrapment of 6kDa secretory antigen in liposomes ranged from 52-54%, which was comparable to 45-50% entrapment, observed with protein antigens employing the same method of preparation (Meyer et al., 1986).

Immunization with 6kDa antigen encapsulated in liposomes, included significant humoral and cell mediated immune responses as determined by ELISA and T-lymphocyte proliferation assay. The augmentation of T and B lymphocyte mediated immune response is in accordance with earlier reports in which liposomes have been used as an adjuvant for Mycobacterial antigens (Pancholi et al., 1989, Chugh and Khuller 1993; Andersen, 1994) and membrane antigens of Leishmania donovani and Entamoeba histolytica (Vinayak et al., 1987; Russell and Alexender, 1998). It is widely believed that
the process of immunological presentation of protein antigens to the immune system involves initial processing of the antigen by professional antigen presenting cells such as macrophages and dendritic cells (Unaue and Cerottini, 1989). The ability of liposomes encapsulated antigens to enhance the immune response is due to the natural targeting of liposomes to macrophages (Shek and Sabistan, 1982; Van-Rooijen and Ven Nieuwmege, 1983a; Beatty et al., 1984). Activated macrophages have been shown to play a major role in defense against infectious organism in vivo and in vitro (Kirsh and Poste, 1986).

The immune response (cellular and humoral) elicited by the 6 kDa secretory protein entrapped in liposomes were similar (Table-XII, VIII and Figure-36, 37) to that elicited by 6 kDa-FIA and BCG vaccine, thus indicating that liposomes can be effectively used to generate strong immune response by the entrapped antigen. Immunization with 6 kDa secretory protein entrapped in liposomes resulted in increased resistance to intravenous challenge with LD_{50} of M. tuberculosis H_{37}Rv 4th weeks post immunization as monitored by survival rates and enumeration of viable tubercle bacilli in the infected organs (spleen, liver and lung). Immunized animals with 6kD-Lip and BCG showed considerably higher (p<0.001) survival rates (76%), (72%) as a compared to control animals (32%) on 30 days post challenge (Table-XIX and Figure - 38).

There was a generalized drop in the number of bacteria isolated from spleen, lungs and liver (Table- XX) as compared to control mice indicated that immunization with 6kDa-Lip ensure better clearance of the microorganism from the three organs (liver, lungs and spleen).
Our findings that liposomes could be effectively used to elicit the desired immune response and confer significant protection in the immunized animals are similar to the observation of other workers who have demonstrated increased immune response (Vinayak et al., 1987; Russell and Alexender, 1998; Pancholi et al., 1989; Chugh and Khuller, 1993) with the liposomal encapsulated antigen. The immunogenicity of the liposomal entrapped antigen used in the study was comparable to that induced by the incorporation of the culture filtrate / extracellular proteins of Mycobacteria in different adjuvant system such as Syntax (SAF1), DDA, RIBI adjuvant, and microsphere (PLG) particles (Andersen, 1994; Horwitz et al., 1995; Vordermeier et al., 1996). These adjuvants have been reported to augments, potent cell mediated immunity with secretion of IFN-γ and generation of IgG a antibodies. DDA, SAF1 and liposomes entrapped antigens have also been shown to impart protection against the disease in animal model (Andersen, 1994; Horwitz et al., 1995). Some recent reports suggest that saponin adjuvant (such as ISCOMS) stimulate a mixed response skewed toward a Th1 response characterized by production of IFN-γ and some IL-5 from primed T cells.

Thus, the use of liposomes to deliver the antigen to generate desired cellular immune response and impart protection appears to be better, as liposomes have the ability to load a large variety of molecules into them regardless of their size, solubility and charge characteristics (Gregoriadis, 1990). The extensive knowledge of liposomal properties, behavior in vivo, the variety of ways of controlling such behavior and their non-toxic nature supports further
efforts towards the application of the liposomes as immunological adjuvant and vaccine carriers.

**Serodiagnostic potential of cocktail of antigens**

Developed ELISA based serodiagnostic test using a cocktail of all five proteins was used to enhance the sensitivity of the assay and attempts were also made to check the specificity of the cocktail.

Out of 148 pulmonary patients, 139 were positive, providing sensitivity of 94.27%. Similarly, out of 50 extra pulmonary patients, 46 were identified positive giving a sensitivity of 92.59%. Sensitivity for meningeal tuberculosis was found to be 60.86% (Table-XXII and Figure- 40).

The results of our study were also compared with a commercially available diagnostic kit containing cytosolic antigen A60 (Table –XXIII and Figure- 41). It was observed that both the sensitivity, specificity of our combination of antigen and A60 was almost similar.