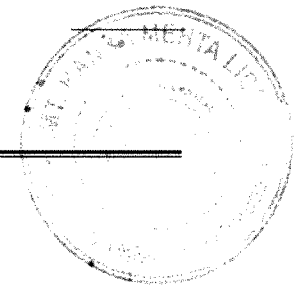

1. Introduction

Tuberculosis (TB) is one of the deadliest scourges of humans, infecting about one-third of the world's population (i.e. 2 billion people). Globally, 9.2 million new cases and 1.7 million deaths occurred from TB, out of this half the cases and one third of the deaths were reported with HIV-TB co-infection (WHO 2008). Tuberculosis disease is caused by infection with organisms of the *Mycobacterium tuberculosis* (*MTb*) complex, which includes *M tuberculosis, Mycobacterium bovis*, and *Mycobacterium africanum. M africanum*, a bacterium that can be transmitted through the air. If it is left untreated, TB can cause the development of cavities in the lungs and other tissues, leading to a variety of symptoms, including severe cough (at times with blood), fever, and weight loss, and might become life-threatening. Growth of the bacteria in the lungs of infected persons is controlled in many cases, but not eradicated, by the immune system. When immunity fails because of malnutrition, aging, or HIV infection, the bacteria grow, causing active TB of the lung (Springett 1990).

TB is prevalent in developing countries of South Africa and Asia. There are three main reasons of TB epidemic include: HIV- AIDS which hampers immune function and support mycobacteria for growth (WHO 2007), emergence of Multidrug-resistant strains, (MDR TB) and Extremely drug-resistant tuberculosis (XDR) (Matteelli 2007), which is difficult to treat with conventional anti-biotic regime and failure of existing BCG vaccine in providing good protection in Adult pulmonary TB (Yasir 2006).

BCG (Bacille- Calmette-Guerine), currently used vaccine for TB provides an excellent protection against disease to the children. However, it shows variable efficacy in Adult- Pulmonary TB disease. Current status of the disease indicates remarkably high numbers of active TB disease where BCG shows limited efficacy. This high increase in Adult- pulmonary TB cases supports need for the development of a therapeutic vaccine or BCG booster vaccine which can control active disease which can enhance protection efficiency of BCG vaccine in adult (Smith 2000, WHO 2008).

In 1993, the WHO focused researcher's attention on combating the disease by declaring TB a Global Health Emergency (Raviglione 1995). Since then, organized efforts were initiated to control TB. In 2000, WHO recommended chemotherapy, notably Directly Observed Therapy, Short-course (DOTS), include combination of three antibiotic, with which a dramatic increase in the effectiveness of TB control was found (observed cure rate was around 85%) (Broekmans 1994, Chaulk 1998). Only 12% of patients with active disease worldwide have benefitted with this therapy. Currently, DOTS require 6–9 months for an effective cure, since it is difficult to kill dormant bacteria with chemotherapeutic drugs. Simultaneously, there is fast and continuous emergence of MDR and XDR TB strain. Many experts of this area believe that for complete eradication of TB, there is a need for a cheap and effective vaccine for mass immunization (Young 2006, Kaufmann 2001). With this intention, a major international research focus on development of new TB vaccines and, as a result, over the last 10 years more than 200 TB vaccine candidates have been tested in mouse, guinea pig and on human primate models (Ginsberg 2000, Orme 2005, Izzo 2005).

The majority of vaccines being developed are designed for parenteral administration. One of the major obvious disadvantages of this delivery method is risk associated with needle-stick injuries particular in high HIV-AIDS prone areas. Another limitation of parenteral immunization is limited mucosal immunity which plays major role in prevention of infectious disease.

The most realistic route for administration of any vaccine is by the route of pathogen entry to the host (Hoggard 2005). Early reports indicated existence of common mucosal immune response, but recent scientific papers (Johansson 2004) stated compartmentalization within mucosal immune system which actually places constraints on the choice of vaccination route for inducing effective immune response at the desired sites. Considering the fact that *MTb.* infections occurs via the respiratory tract and affect the respiratory tract primarily, 75% cases of TB are of pulmonary tuberculosis and extra- pulmonary infection spreads from lung. Hence, intra-nasal route would be the ideal route of vaccination for the development of a mucosal TB vaccine (Giri 2008).

The intra nasal route of administration offers following advantages:

- ✓ Much lower doses of antigen required as there is no significant dilution of vaccine formulation in nasal fluid.
- ✓ No exposure to low pH
- ✓ No exposure to secreted digestive enzymes
- ✓ Nose is easily accessible, highly vascularized and contains numerous microvilli covering the nasal epithelium providing a large absorption area
- ✓ Moreover, intra nasal immunization provides both systemic and mucosal immunity
- ✓ Delivery of Dose without needles; hence needle stick side effects can be minimized.

There have been a number of "new" approaches to the development of improved TB vaccines: DNA vaccines (Lowrie 1997), attenuated strains of Mycobacterium tuberculosis (Guleria 1996) and the use of secreted proteins from *M.Tb.* culture filtrates in new vaccine formulations (Andersen 1992, Singh 1995, Brandt 2002) have all been evaluated.

Early secreted antigenic target (**ESAT-6**), which was isolated from a highly stimulatory low-molecular-mass fraction of short-term-culture filtrate, was the one used for this study. Reports indicated that ESAT-6 is strongly immunogenic, able to elicit antigen-specific T-cell response and provide protective immunity comparable to that achieved with BCG. It is interesting to note that this antigen is expressed in the case of *MTb.* Infection but not by BCG administration (Brandt 2002).

Another protein, Antigen 85B belongs to the group of **Ag-85 complex (A-C),** 30-32 KDa proteins that acts as mycolyl transferases. Because of thier key role in cell wall biosynthesis and extension prior to fission, they are made in copious amounts, particularly when the bacterial culture is in log phase. These proteins have been reported to be leading vaccine candidates by number of workers (Orme 2006, Girard 2005, Yadav 2001, Brookes 2001). Ag85 has been widely studied in mouse primarily as a DNA vaccine (Ulmer 1997) and Olsen et al (2001) have shown promising results using purified proteins.

Many researchers have reported their work with these two proteins individually; i.e. either ESAT-6 alone (Aagaard 2009) or Ag-85B alone (Brooks 2001), or in combination [ESAT-6 and Ag-85B] (Dietrich 2006) or as fusion protein (Olsen 2001). Some researchers have also explored mucosal route for vaccination with these proteins (Carpenter 2005), which is supposed to be the realistic route for TB vaccine administration.

The main problems in formulating these protein based antigens are:

1. Poorly immunogenic in nature when administered mucosally.

   Probable solution could be selection of proper delivery system and adjuvant (Singh 2002, Vajdy 2005) which may help in following way:

   - ✓ Increase immunogenicity
   - ✓ Enhance speed and duration of the immune response
   - ✓ Modulate antibody avidity, specificity, isotypes or sub-class distribution
   - ✓ Promote the induction of mucosal immunity
   - ✓ Decrease dose of antigen in the vaccine
   - ✓ Help to overcome antigen competition in combination vaccine

2. Antigen must reach the epithelia to get presented and interact with Antigen Presentation Cells (APCs) (Singh 2002). To achieve this specific aim, particles loaded with antigens must transport through the epithelia. Longer, sustained stay of such particles at epithelium is equally important to cross the epithelium barrier for eliciting a strong immune response.

   Therefore, a suitable delivery system is very important to achieve both the aims (Giri 2008).

Biodegradable Poly Lactide (PLA) and Poly Glycolide (PGA) and combination polymers Poly Lactide-co-Glycolide (PLGA) have been used in humans for many years as suture and controlled release devices. The ability of particles prepared from these polymers to enhance immune responses to entrapped antigens delivered by mucosal routes is consequence of their uptake into M cells and ability to target mucosal surfaces (Tamber 2005). Most of the work with protein based antigens has been reported with polymer encapsulation. However, surface presentation of antigens would give better immune response as they come directly in contact with antigen presenting cells (APCs). PLA, PLG and PLGA polymers lack reactive groups that can

be used to couple biological molecules for surface presentations (Desai 1991, Barrera 1993, Langer 1993, Langer 1998). Hence, to add reactive group to PLA polymer, we synthesized PLA-PEG-Biotin polymer where PLA is the backbone that provides structural integrity (Salem 2001), PEGylation of PLA helps for mucus penetration (Hanes 2005, Suh 2007, Lai 2009) and Biotin helps for covalent conjugation of protein based antigens. The major outcome of this modification of polymer could bring better immune response without compromising antigenicity of the proteins.

1.1 Aims and objectives of the study

- The aim of the proposed study was based on the reports on mass spectroscopy analysis of Short Term Culture Filtrate (STCF) collected from the infected mice and patients with minimal TB, that have showed presence of isolated antigens. Among the isolated antigens, which are strongly recognized by peripheral blood mononuclear cells are ESAT-6, Ag 85 complex (B), MPT 51, TB 10.4, Mtb 8.4 (Orme 2005). The proposed study was aimed to develop vaccine formulation which can be administered **intra-nasally** with ESAT-6 and Ag85B with suitable adjuvant which can help to increase immunogenicity of these proteins based antigens, for better or equivalent disease protection when compared to the BCG protection.
- The objective of the study was to facilitate direct interaction of antigens with antigen presenting cells (APCs) to achieve comparable immune response when given through Nasal route.

1.2 Hypothesis of the Study

The hypothesis of the present study was based on the fact that the antigens can be better recognised by the body when they interact directly with APCs. The study was also based on the fact that the surface presentation of antigens would be better recognized as compared to that when the antigens are encapsulated in polymeric particles. We also took into consideration reports indicating that the mucosal immunity can give better disease protection than systemic immunity and that Nano-particles (in the range of 200-500 nm) fabricated from biodegradable polymers are more suitable than micro-particles for surface presentation of antigens and subsequent interaction with APCs.

Based on the above inputs, we concluded that to achieve mucosal immunization; the formulation should preferably be given through intra-nasal route (the way pathogen intrudes the host), antigens must cross the mucosal barrier and presented on the surface of the polymers for direct interaction with APCs.

When nano-particles are given through mucosal route, they will localize lymphoidal tissues and stimulate M cell for the production of the specific antibody. Due to the size of the particles, the uptake by lymphoid cells will be immediate and surface presentation of the antigens will facilitate interaction with APCs. Thus, the system would provide efficient and effective immunization.

1.3 Plan of work

- ✓ Literature review
- ✓ Selection and procurements of antigens
- ✓ Selection of Polymers and synthesis of polymer
- ✓ Analytical techniques
- ✓ Preparation of Nano particles and characterization
- ✓ In-vitro Evaluation of the formulation for mucus penetration
- ✓ Stability studies
- ✓ In-vivo evaluation

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Reviewer's Comments: