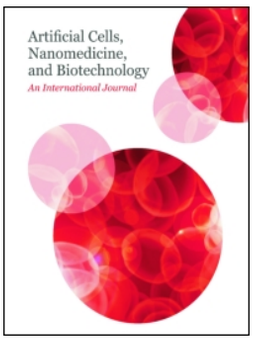

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Development of an effective delivery system for intranasal immunization against *Mycobacterium tuberculosis* ESAT-6 antigen

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ABSTRACT

Introduction The early secreted antigenic target 6-kDa protein (ESAT-6) plays an important role in immune protection against Tuberculosis. Owing to its great potential to increase immune response, chitosan can be considered as a suitable biodegradable polymer for intranasal administration. **Methods** The physiochemical properties of the nanoparticle were measured *in vitro*. Two weeks after the last intranasal administration, blood samples were collected and specific IgG, IFN-gama, and IL-4 levels were measured by ELISA. **Results** Chitosan nanoparticles containing ESAT-6 demonstrated stronger ability to induce IFN-gama, IL-4, and IgG antibody level than the control groups. Conclusion Administration of chitosan nanoparticles can be a suitable method to induce more appropriate immune responses against low inherent immunogenic tuberculosis proteins through intranasal routs.

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Chitosan; cytokines; ESAT-6; immune responses; *Mycobacterium tuberculosis*

Introduction

Tuberculosis (TB), as a worldwide health problem, remains the leading cause of infection and mortality worldwide (Kochi 1994, Ravn et al. 1999, Skeiky and Sadoff 2006). Taken together, vaccination is the best strategy for the control of tuberculosis (Wang et al. 2007). However, Bacillus Calmette–Guérin (BCG), as a first-generation TB vaccine, has been used for almost 90 years. The protective efficacy of BCG vaccination has varied in different populations (Fine 1995).

The inability of BCG to induce protective responses against pulmonary tuberculosis has led to new efforts in the development of next-generation TB vaccines (Young and Stewart 2002). Subunit vaccines which were developed on the basis of protective antigens demonstrated high levels of specificity and safety, suggesting them as being appropriate candidates to overcome many problems associated with other types of vaccines (Andersen 2001, Babiuk 1999, Brown 1992, Levine and Sztein 2004). The Early Secreted Antigenic Target 6 kDa protein (ESAT-6), encoded by a gene in the RD1 region, is one of the best candidates for TB subunit vaccines. Although absent in BCG and vaccinated persons, ESAT-6 is expressed in *M. tuberculosis* and recognized by the immune system in TB patients (Brandt et al. 2000, Harboe et al. 1996, Mahairas et al. 1996). However, it is well established that ESAT-6 has a low inherent immunogenicity, requiring co-administration with a potent immunostimulatory adjuvant to induce efficient and specific immune responses (Brandt et al. 2000, Black et al. 2010). Encapsulation of antigens in nanoparticles has been considered as a suitable delivery strategy for the mucosal administration of vaccines. This could increase the stability of substances, interact with hydrophilic and hydrophobic substances, increase the residence time of antigens, thereby resulting in an increase in antigen uptake by M cells connected to the nasal-associated lymphoid tissues (NALTs) (Gelperina et al. 2005, Nagamoto et al. 2004, Slütter et al. 2010). Among the different polymer candidates used in nanoparticle synthesis, Poly lactic-*co*-glycolic acid (PLGA) and Chitosan seem to be the more appropriate ones (Slütter et al. 2010). However, N-trimethyl chitosan (TMC), which is a derivative form of chitosan, is preferred to PLGA, due to its low toxicity, water solubility, positive charge, and muco-adhesive properties (Amidi et al. 2010). The purpose of this study was to demonstrate whether intranasal administration of ESAT-6 in TMC can promote and build up its inherent low immunogenicity.

Materials and methods

Synthesis of ESAT-6 nanoparticles

ESAT-6 nanoparticles were prepared based on ionic gelation of Trimethyl Chitosan (TMC), previously developed (Slütter et al. 2010). Briefly, various concentrations (0.5, 1, 1.5, and 2 mg/ml) of Trimethyl Chitosan (Merck, Germany) were prepared in distilled water and filtered through 0.45 -μm membrane filters. The ESAT-6 recombinant protein (10 μg/ml) was added to sodium tripolyphosphate (TPP) solution (1 mg/ml), and 0.2 mL

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of ESAT-6/TPP solution (1 mg/ml) was added to TMC solution on magnetic stirrer at room temperature for 40 minutes. A separation of the synthesized nanoparticles was carried out in the presence of glycerin (10 μl), by centrifugation at 10,000 g for 30 minutes, followed by washing and finally subjected to physicochemical analysis.

Physicochemical characterization of ESAT-6 nanoparticles

The size and zeta potential of the synthesized nanoparticles were determined by dynamic light scattering (DLS) using a zetasizer SZ3000 (Malvern instrument, Worcestershier, UK.) (Slütter et al. 2010). The morphology of the nanoparticles was examined by transmission electron microscopy (TEM); determination of the encapsulation efficiency of ESAT-6-loaded TMC nanoparticles was done by separating the particles from the preparing media, followed by centrifugation at 10,000 g for 30 minutes. The amount of free ESAT-6 proteins in supernatant was measured by using the micro bicinchoninic acid (BCA) method (Sigma-Aldrich, Germany) according to the manufacturer's instructions (Slütter et al. 2010). The encapsulation efficiency (EE) of the nanoparticles was calculated by the Equation given as:

$$\%\mathrm{EE} = \frac{\left\{\begin{array}{c}(\text{Total amount of ESAT-6})\\ -(\text{Free ESAT-6 in the supernatant})\end{array}\right\}}{\text{Total amount of ESAT-6}} \times 100$$

Finally, the particles were re-suspended in phosphate buffer saline (PBS, pH 7.4) for intranasal immunization. The final concentration of ESAT-6 in TMC particles was adjusted to 30 μg/10 μl.

Experimental groups and immunization

Six- to 8-week-old female BALB/c mice were obtained from the local animal facility (Razi Vaccine and Serum Research Institute, RVSRI). The mice were maintained under controlled environmental conditions and allowed access to food and drink ad libitum. Mice were randomly divided into four groups; mice in groups 1–4 were intranasally immunized three times at 2-week intervals with 30 μg of ESAT-6-loaded TMC nanoparticles, ESAT-6, TMC, and PBS, respectively (Brandt et al. 2000). Prior to immunization, mice were slightly anesthetized by intraperitoneal injection of a ketamine/xylazine cocktail. Fifteen days after the last priming dose, blood samples were collected by retro-orbital punctures. The Sera were isolated by centrifugation and stored at −20 °C for further analysis.

Lymphocyte culture and cytokine determination

Two weeks after the last immunization, all experimental mice were scarified by cervical dislocation, and the spleens dissected. The splenocytes were prepared as a single cell suspension, cultured (2×10^5 cells/well) in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) in the presence of antigens (5 μg/ml of purified ESAT-6), and incubated for 72 hour at 37 °C in an atmosphere of 5% CO_2 (Brandt et al. 2000). Positive controls were stimulated with phytohemagglutinin-A (PHA) (2 μg/ml) and negative controls were left unstimulated. Cell culture supernatants were harvested for cytokine determination by murine IL-4 and IFN-γ ELISA kits (R&D Systems Inc., Minneapolis, MN) as noted in manufacturers' instructions.

Evaluation of ESAT-6-specific antibody responses in serum

The levels of specific antibodies against ESAT-6 were determined by an indirect ELISA method as previously described (Tebianian et al. 2011). Briefly, 96-well flat-bottom ELISA plates (Nunc, Denmark) were coated overnight at 4 °C with 100 μl of coating buffer (0.1 M carbonate/bicarbonate, pH 9.6) containing the purified ESAT-6 protein (0.2 μg/well). After washing the plates with PBS containing 0.05% of Tween 20 (PBS-T), blocking was carried out with 300 -μl/well of blocking buffer (3% non-fat dry milk powder and 0.2% Tween-20 in PBS) for 2 hours at 25 °C. The mouse serum samples (1/100 diluted) were added to each well, followed by incubation of the plates for 60 minutes at 37 °C and finally washing with PBS-T. Hundred microliters of HRP-conjugated goat anti-mice IgG (Abcam, 1:6000 diluted) was added to each well, and the plates were incubated for 60 minutes at 37 °C. After additional washing steps, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrates were added to each well. Upon 20-minute incubation at room temperature, the stop solution (0.1 N sulfuric acid) was added, and optical density was measured at 450 nm using an automatic microplate readder (Bio-Rad, USA).

Statistical analysis

Data were reported as the mean ± standard error of the mean (SEM). Differences between groups were performed using unpaired two-tailed Student's *t*-test. A difference was considered to be statistically significant for *p* values of lower than 0.05 ($P < 0.05$).

Results

Size and zeta potential and morphologic characterization of the nanoparticles

Particle size and morphological characterization of ESAT-6 nanoparticles were measured using the DLS technique and TEM, respectively. According to the obtained results, the mean of the particle size was 242.8 ± 8.71 with a zeta potential of 29 ± 8/71 mV which were acquired in a 1-mg/ml TMC concentration (Figure 1). It seemed that the mean of the ESAT-6 particle size and zeta potential was elevated by an increase in the TMC concentration. Furthermore, the polydispersity index (PI) values correlated with the variation in particle size distribution, indicating that the samples prepared with the 1-mg/ml TMC concentration possessed narrow size distribution than those with higher and lower TMC concentrations. The loading efficacy of nanoparticles was measured by the bicinchoninic acid protein assay above 95% (data not shown). After 200 hours, the *in-vitro* antigen release from nanoparticles was detected as 67.5%.

The effect of nanoparticles on IFN-gama and IL-4 production

To evaluate ESAT-6-formulated TMC nanoparticles encapsulated in a cytokine pattern, the release of IFN-γ and IL-4 was measured by re-stimulated splenocytes, using the ELISA method. As shown in Figure 2, animals immunized with ESAT-6, with or without the TMC nanoparticle formulation, elicited a strong IFN-g response. However, the nanoparticle form induced the higher levels of IFN-g responses in comparison with the pure ESAT-6 recombinant protein. Additionally, the significant amount of ESAT-6-specific IL-4 production was detected in splenocyte cultures of animals immunized by ESAT-6 and ESAT-6/TMC nanoparticles. In addition, ESAT-6-containing chitosan nanoparticles had a much higher ability to induce IL-4 secretion (Figure 3). Overall, the results revealed that the properties of ESAT-6-containing chitosan nanoparticles induced the immune system in order to produce IFN-gamma and IL-4 cytokines.

Table 1. The effect of TMC concentrations on the mean particle size, PI, and zeta potential of the ESAT-6-encapsulated nanoparticles

Polydispersity index	Average size (nm)	Zeta potential (mV)	TMC (mg/ml)
0/202 ± 0/058	227/18 ± 9/58	21/ 22 ± 4/05	0/5
0/165 ± 0/053	242/46 ± 8/71	29/0 ± 3/5	1
0/222 ± 0/081	284/98 ± 11/54	36/3 ± 4/31	1/5
0/584 ± 0/091	485/57 ± 26/22	38 ± 4/84	2

Antibody responses to recombinant ESAT-6 proteins

To investigate the efficiency of ESAT-6-containing nanoparticle in the induction of humoral immune responses, antigen-specific antibody responses were examined in mice

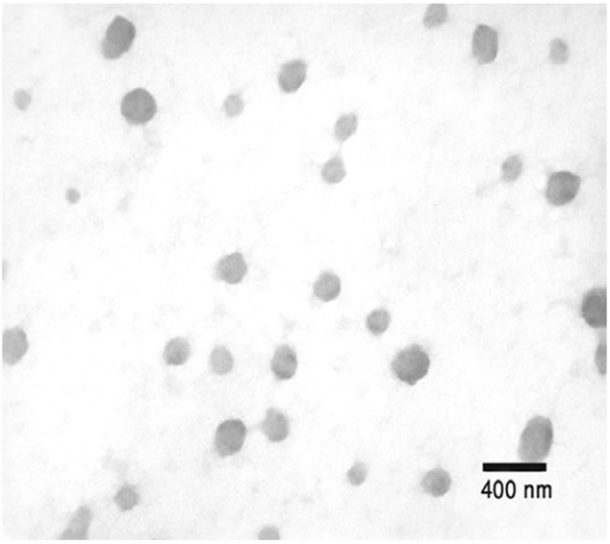

Figure 1. The TEM micrograph of ESAT-6 encapsulated in TMC nanoparticles.

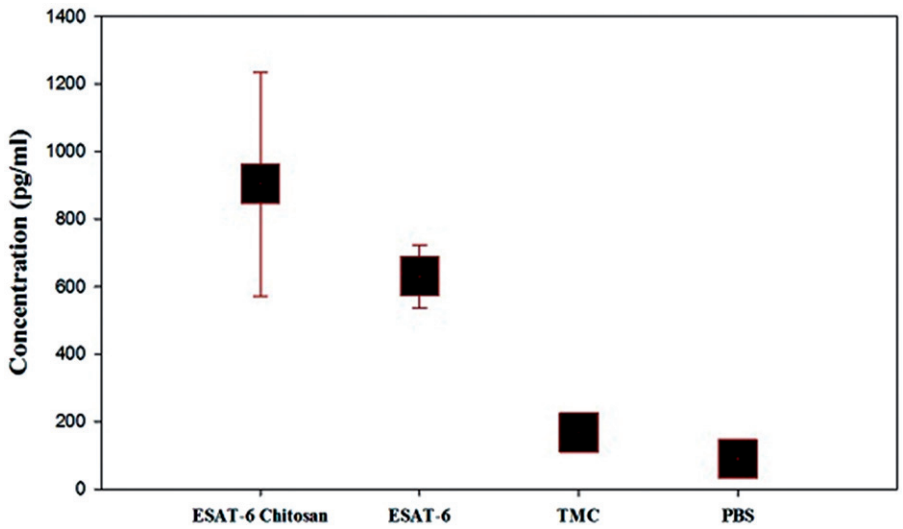

Figure 2. Interferon gamma releases from splenocytes of immunized mice. Values represent the mean ± standard deviation of five mice per group.

immunized with purified ESAT-6, with or without the TMC nanoparticle formulation. Two weeks after the last immunization, the specific IgG antibody titers against ESAT-6 were evaluated in the sera of immunized mice. The mice immunized with the ESAT-6 protein or ESAT-6-containing chitosan nanoparticles yielded higher levels of the specific IgG antibody when compared with other control groups. Moreover, the responses of the antibody were significantly higher in animals that received ESAT-6 in TMC nanoparticles than those that received noncapsulated ESAT-6 (Figure 4).

Discussion

It has been demonstrated that the administration of intranasal vaccine has many profits and advantages when compared with other routes. Draining lymph nodes of the nasal cavity conduct antigens to blood circulation without first-pass metabolism. This leads to initiate a systemic immune response, even in distant organs, such as lung (Jabbal-Gill 2010).

Despite global efforts to control TB in recent years, it has remained as a serious mortal disease worldwide (Black et al. 2010). However, subunit vaccines are important vaccine candidate for the prevention of TB. Among different *M. tuberculosis*

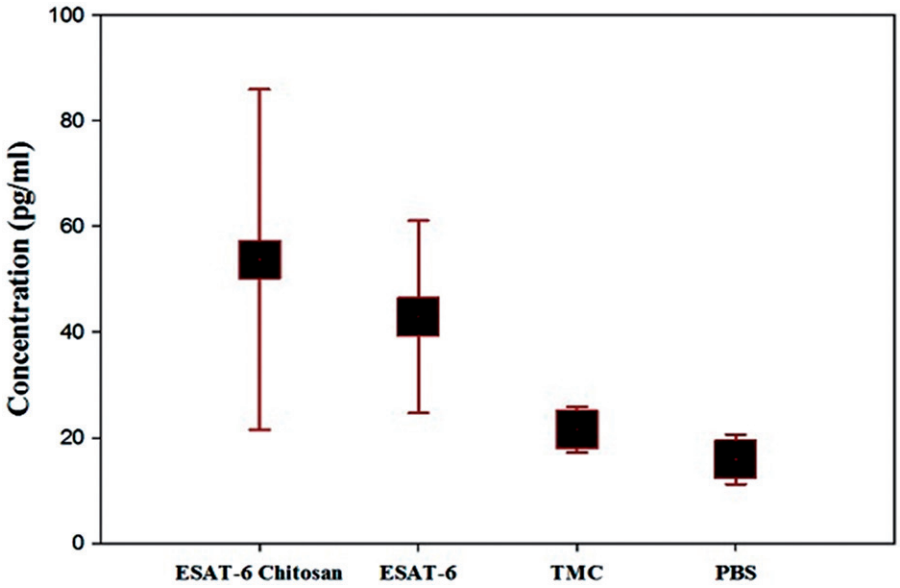

Figure 3. Interleukin 4 releases from splenocytes of immunized mice. Values represent the mean ± standard deviation of five mice per group.

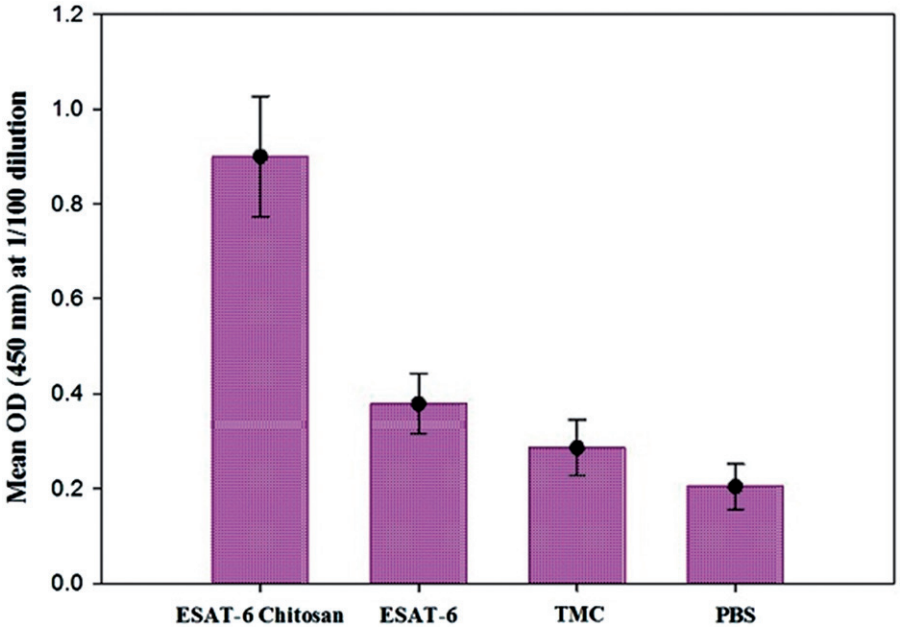

Figure 4. The levels of anti ESAT-6 specific antibody in different groups.

antigens, ESAT-6 has been found broad use in many studies to stimulate specific immune responses (Henson et al. 2014, Soleimanpour et al. 2015). Because of the poor ability of subunit vaccines to induce a potent immune response, nanoparticles are currently being considered as suitable agents to induce a strong immune response (Vila et al. 2004). Nanoparticles are constructed by a lot of biodegradable polymers, such as chitosan and PLGA (Joshi et al. 2013). The TMC polymer exhibits exceptional adjuvant properties, including slow release of antigens, cytoskeleton change, cell-junction opening by interaction with zo-1 proteins and an increase in the intranasal residence of ESAT-6 by positive charge on the nanoparticles, thus decreasing the clearance of ESAT-6/TMC nanoparticles (Shaji et al. 2010). Furthermore, a variety of studies have indicated that chitosan shares a positively charged property which serves as a significant factor for the use of chitosan as a more effective carrier than PLGA (Slütter et al. 2009). Since the mean ESAT-6/TMC nanoparticle size obtained in this study was 245 nm, the nanoparticles can be easily absorbed by phagocytes. Previous researches demonstrated that nanoparticles of mean size smaller than 1 μm could be absorbed by dendritic cells (DC) (Nagamoto et al. 2004). Jiskoot et al. emphasized that nanoparticle size between 200 and 500 nm demonstrated a minor difference in stimulating immune responses by intranasal administration (Gutierro et al. 2002, Slütter et al. 2010). Therefore, the nanoparticle size acquired in this study has a suitable and homogeneous size which could to be absorbed by DC and also triggers the immune response.

The positively charged ESAT-6/TMC nanoparticles offer several advantages over the noncapsulated ESAT-6; whereas non-capsulated ESAT-6 has a negative charge and is cleared in a short time, positive charge on ESAT-6/TMC can affect the nanoparticle adsorption process because positively charged nanoparticles interact with the negatively charged mucus, increase the residence time of ESAT-6/TMC on the mucus, induce a better uptake of the ESAT-6 antigen by DCs, and elicit a stronger immune response (Huang et al. 2004). Interestingly, following the adsorption of nanoparticle to the mucus layer, the M cell related to nasopharynx-associated lymphatic tissue (NALT) uptakes encapsulated nanoparticles by the transcytosis mechanism, and transfers the antigens to the DCs below, without any degradation (Illum et al. 2001). The TMC ability in the maturation of immature DCs has been described by a variety of studies (Reddy et al. 2006, Slütter et al. 2009). As a result, following the migration of matured DCs to the lymph nodes and antigen presentation to the T cells through MHCII, T cells diverge to Th2 and Th1, eliciting humoral and cellular immune responses, respectively. In a variety of reports, TMC has been mentioned as a mucosal adjuvant with great impact on TH1/TH2 regulation, depending on the antigen and rout of administration (Jabbal-Gill 2010, Li et al. 2009, McNeela et al. 2004, Porporatto et al. 2005). Although immunity against TB infection is related to cellular and humoral immunity, in this case the cellular immunity demonstrates critical roles. Rauw et al. emphasized that the involvement of the TMC adjuvant promoted the cellular immune response and secretion IFN-gama (Ghendon et al. 2009, Rauw et al. 2010). Because of the proton scavenger property of TMC, It is possible that antigens leave the APC endosome to cytoplasm and enhance antigen presentation by MHCI in DCs (Slütter et al. 2009, Strong et al. 2002). In this way, the chitosan may play a significant role in increasing the immunity against TB infection. In the current study, similar those acquired by Carpenter et al., it was demonstrated that mucosal administration of ESAT-6/TMC nanoparticles was able to induce both Th1/Th2 cells and. (Carpenter et al. 2005).

Several studies indicated the principal function of humoral immunity against TB infection. In one study, it was indicated that IgG played a protective role in the prevention of disseminated TB in children (Zuñiga et al. 2012). Interestingly, in another study, it was demonstrated that there existed a relation between the IgG titers against active TB and those against antituberculin to forestall the reactivation of TB in high-risk populations (Encinales et al. 2010). Balu.S et al. also succeeded to exert monoclonal IgA anti- α-crystallin for the reduction of infection by H37Rv Mtb in transgenic mice (Balu et al. 2011). Several strategies were participated in the prevention of Mtb infection by antibodies. The main mechanism of this strategy is to inhibit the attachment of Mtb to the host cell surface and slap dawn of infection. Another important strategy is developed based on the interaction between lysozyme and phagosome vesicles and promoting of their fusions (Armstrong and Hart 1975, Encinales et al. 2010). Therefore, this study emphasizes a successful carrier role of TMC to induce T-cell differentiation and to promote the immune responses in TB infection. Moreover, further studies are required to evaluate other immunological features of ESAT-6 encapsulated in TMC as a novel and efficient vaccine candidate to prevent TB.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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