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ORIGINAL RESEARCH PAPER

Chitosan (CHT) and trimethylchitosan (TMC) nanoparticles as adjuvant/ delivery system for parenteral and nasal immunization against *Mycobacterium tuberculosis* (*MTb*) ESAT-6 antigen

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ABSTRACT

Objective(s): An efficient vaccine against TB is an urgent need. TB peptides are safe candidate but they are weak immunogens and needs to be potentiated by adjuvant/delivery systems. The main purpose of the present study was to determine the potential of CHT based NPs containing ESAT-6 antigen of *M. tuberculosis* for inducing mucosal and systemic immune responses after intranasal and subcutaneous injection in mice model.

Materials and Methods: CHT and TMC based NPs were prepared by coating of cationic polymer on the anionic peptide by ionic gelation method and their characteristics were evaluated by scanning electron microscopy (SEM) and dynamic light scattering (DLS). Physical stability of NPs was studied within 30 days. Finally, the ability of formulated NPs to elicit immune responses in BALB/c mice were evaluated following nasal and subcutaneous immunization.

Results: The best weight ratio of antigen to polymer (CHT or TMC) was 1:2. CHT and TMC NPs had a mean size of 356.3 ± 42.20, and 470.3 ± 48.21 nm, respectively. NPs were stable up to 15 days. CHT:ESAT-6 NPs gave higher serum IgG1 and IgG total responses and TMC:ESAT-6 NPs induced high titers of IgG2a and IFN-γ.

Conclusion: Regards to the importance of cellular immune responses in effective protection against TB, and also the solubility in physiological pH, TMC NPs are more efficient adjuvant/antigen delivery system for immunization against TB.

Keywords: *Chitosan and Trimethylchitosan nanoparticles; ESAT-6 antigen; Mycobacterium tuberculosis; Nasal immunization; Subcutaneous immunization*

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INTRODUCTION

Tuberculosis (TB) is an important problem in terms of public health in the world due to morbidity and mortality in numerous countries during the history [1]. Many vaccine researchers are believed that the most important method to prevent from TB is the existence of an effective vaccine, especially in developing countries with the low resources and high incidence of MDR-TB [2, 3]. Until now, only effective vaccine against TB infection is *Mycobacterium bovis* Bacillus Calmette-Guerin or BCG, but its protective efficacy rate is controversial, ranging from 0 to 85% in different regions [4, 5]. There are many reports about recombinant antigens of *Mycobacterium*

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tuberculosis (*M. tuberculosis*) that are attractive as potential vaccine candidates. These are among alternative strategies for the development of future TB vaccines [6]. Among many immunodominant protein antigens of TB, ESAT-6 antigen, the 6-kDa early secretory antigenic target 6, which is expressed in the early step of TB infection and could be strongly identified by Th1 cells and induce high production of IFN-γ and IL-12 cytokines against *TB* [5, 7, 8]. But, inadequate stimulation of the innate immunity is one of the main drawbacks of these types of vaccines. Therefore, an efficient adjuvant/delivery system is required for potentiating of immune responses against these antigens [3]. Natural polymers, chitosan (CHT) and its derivatives, are non-toxic, biodegradable and biocompatible polymeric materials that have been used for delivery of peptide, protein and drug and also for vaccine development [9].

These cationic polysaccharides have many advantages for immunization against TB, including: 1) strong stimulation of mucosal and systemic immune responses, 2) giving particulate nature to the soluble antigens and better presentation to the APC cells and also to the mucosa associated lymphoid tissue (MALT) microfold (M) cells, and 3) efficient mucosal vaccine delivery because of its cationic, mucoadhesivity and nontoxicity properties [9, 10]. As TB pathogen enters the body through the mucosal surface of the respiratory tract, therefore, creating appropriate mucosal as well as systemic immune responses at the site of pathogen entry is important [10, 11]. Therefore vaccination against TB by nasal route is great importance.

The main purpose of the present study was to determine the potential of CHT based NPs containing ESAT-6 antigen of *M. tuberculosis* for inducing mucosal and systemic immune responses after intranasal and subcutaneous injection in mice model.

MATERIALS AND METHODS

Materials and animals

ESAT-6 protein expression and purification was previously performed by Dr. M. Sankian (Mashhad, Iran). Chitosan was purchased from Primex (Avaldsnes, Norway). TMC with a degree of quaternization of 23.8% was synthesized from 92% deacetylated (MW 120 kDa) chitosan and characterized by NMR [12]. Goat anti-mouse IgG, IgG1 and IgG2a conjugated with horseradish peroxidase were obtained from Zymed laboratories (San Francisco, USA). ELISA assay kit for mouse IFN-γ and IL-4 cytokines were bought from Mabtech AB (Sweden). Goat Anti-Mouse IgA-HRP was purchased from Invitrogen (Invitrogen, USA). BCA protein assay kit was obtained from Pierce (Thermo Scientific, Rockford, IL). Female BALB/c mice, aged 6–8 weeks, were purchased from Pasteur Institute (Tehran, Iran) and maintained in animal house of Pharmaceutical Research Center. The mice were fed with tap water and standard laboratory diet (Khorassan Javane Co, Mashhad, Iran). Mashhad University of Medical Sciences, Ethical Committee Acts was basis of all animal experiments.

Preparation of CHT: ESAT-6 and TMC: ESAT-6 NPs

CHT: ESAT-6 and TMC: ESAT-6 NPs were prepared by addition of equal volumes of polymer solution to ESAT-6 dispersion and pipetting for about 5 s [12]. 50 µl of CHT or TMC solution in PBS buffer (pH 6.7, 8 mM) with different concentrations were added and mixed with 50 µl of ESAT-6 (0.1 mg/ml) dispersions. Six antigen/polymer ratios (0.5:1, 1:1, 1:2, 1:4, 1:6, and 1:8) were mixed for preparation of NPs.

Characterization of NPs

Physical characteristics of CHT*:* ESAT-6 and TMC: ESAT-6 NPs including size, zeta-potential and polydispersity index (PDI) was studied by dynamic light scattering (Zetasizer Nano, Malvern Instruments, Malvern, UK). The physical stability of NPs was also studied for 30 days. Each NP formulation was prepared in triplicate and NPs were kept at 4 °C. Each five days, physical characteristics of NPs in terms of size, PDI and zeta potential were evaluated.

Immunization of mice

Mice (6 animals per group) were immunized either nasally or subcutaneously with different formulations as follows: group 1) ESAT-6 dispersion (nasal), 2) ESAT-6 dispersion (s.c), 3) CHT:ESAT-6 NPs (nasal), 4) CHT:ESAT-6 NPs (s.c), 5) TMC:ESAT-6 NPs (nasal), 6) TMC:ESAT-6 NPs (s.c), 7) PBS solution (nasal), and 8) PBS solution (s.c). For nasal administration, mice were anesthetized using 200 ìl of anesthesia solution containing Xylazine (10 µl), Ketamine (10 µl) and 164 µl of normal saline. ESAT-6 protein dose used for vaccination was 25 µg per mouse per injection. Three injections of formulations were performed at days 0, 14 and 28.

Immunoassay

In the sixth week after the first immunization, evaluation of immune responses was carried out by taking blood samples, nasal lavage samples, and removing the spleens. To obtain the sera, blood samples were centrifuged at 3500 rpm for 20 min. In order to obtain nasal lavage samples, mice were sacrificed and samples were collected after washing the nose with 1 mL PBS. Blood and nasal lavage samples were stored at -20 °C until use. For cytokine assay, extracted spleen cells from mice were centrifuged at 1200 rpm for 5 min, and then ammonium chloride was added for the lysis of RBCs. The cells were cultured in RPMI (Sigma, USA) solution and incubated in a humidified 5% CO_2 incubator for 72 h at 37 °C. After 72 h, supernatants were collected and immediately were used for measuring of the cytokines. To assess the anti ESAT-6 IgG subtypes antibodies in the sera and sIgA antibody in nasal lavage of mice, the direct ELISA method and in order to measure cytokines in the supernatant of cultured spleen cells, indirect ELISA method were used.

Statistical analysis

Data analysis was assessed by one-way analysis of variance (ANOVA) via Tukey-Kramer test. If P value was lower than 0.05, it was considered as level of statistically significant. To further ensure all measurements were repeated at least three times and were presented as mean ± SD.

RESULTS

Characterization of NPs

In the present study, particle size, zeta potential and PDI of M. tuberculosis ESAT-6 protein were determined as 336.3 nm, -6.78 mV and 0.50, respectively.

The negative charge of ESAT-6 antigen facilitates the electrostatic attachment of the cationic polymer on the antigen. Characteristics of CHT based NPs are listed in Table 1. Based on these data, the best ratio of ESAT-6 to CHT and TMC polymers is 1:2. It's due to minimum particle size and maximum zeta potential in this ratio. It was found that with increase of polymer, zeta potential will increase and particle size will decrease. In agreement with the other studies, in similar conditions, CHT NPs showed lower positive charge than TMC NPs.

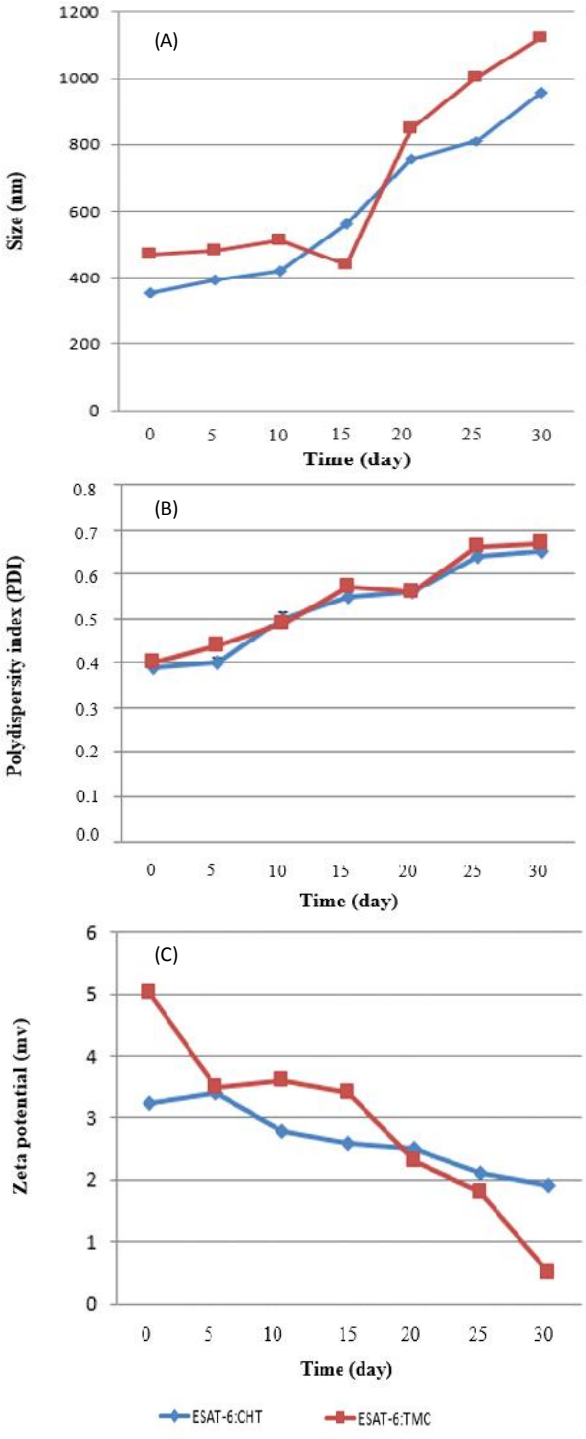

Fig. 1. The physical stability of NPs during a month based on, A) particle size (nm), B) polydispersity index (PDI), and C) zeta potential (mv)

The physical stability of NPs based on their particle size, PDI and zeta potential were studied for 1 month (Figs. 1). Both kinds of NPs showed a nearly same trend of instability. For both NPs, size (from about 400 nm to about 1000 nm) and PDI (from about 0.4 to about 0.7 nm) were increased during one month, which could be resulted from NPs aggregation. The zeta potential was also decreased from about 4 mV to about 1.5 mV. This could be attributed to detachment of polymer coat during the study. This decrease of positive charge allows more attachment of NPs and results in bigger size and PDI.

Immunological responses

After three immunizations, the antibody titers in sera and nasal lavages of immunized animals were determined by an ELISA method. As can be seen in Fig 2, the highest IgG1, IgG2a and total IgG antibody titers were observed in mice immunized with ESAT-6:CHT and ESAT6:TMC NPs via subcutaneous route. This reflects the stimulating effect of the NPs are higher than the ESAT-6 antigen alone ($P<0.01$). Also, intranasal administration of ESAT-6: CHT NPs showed the highest IgA titers ($P\geq0.05$) (Fig. 3). After intranasal administrations, low IgG1, IgG2a and total IgG titers were observed compared with parenterally immunized animals ($P<0.01$). The highest IgG2a /IgG1 ratio was observed after nasal administration of ESAT-6: TMC NPs. Concentration of IFN-γ in supernatant of cultured spleen cells of immunized mice with different formulations was determined by an indirect ELISA method (Fig. 4). The highest IFN-γ concentration was observed in mice subcutaneously immunized with TMC: ESAT-6 and CHT: ESAT-6 NPs ($P<0.001$).

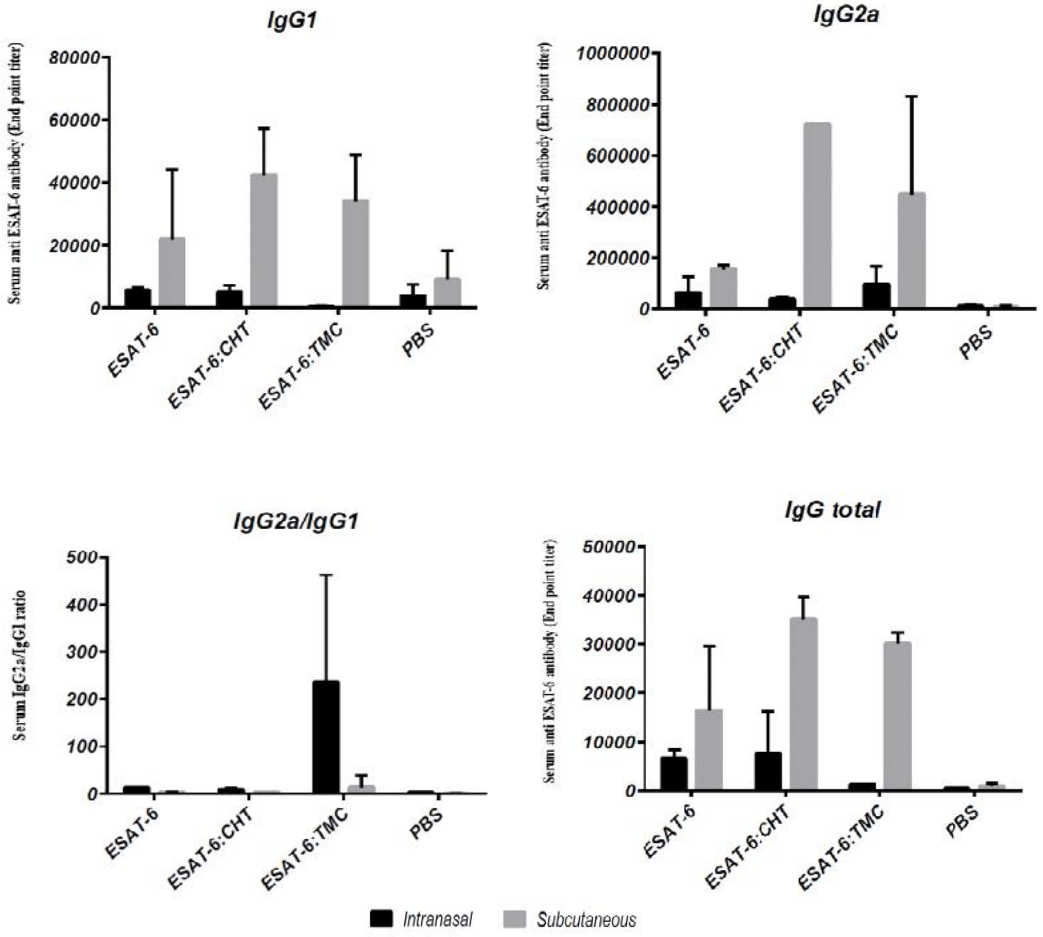

Fig. 2. Serum anti ESAT-6 antibody titers. Mice were immunized i.n. and s.c. with different formulations in days 0, 14 and 28. In the sixth week after the first immunization, mice were bled and antibody titers were assessed by an ELISA method

Table 1. Characterization of CHT and TMC NPs

Formulation	n	Ratio	Z. average(nm)	Polydispersity Index (PDI)	Zeta Potential (mV)
ESAT 6:CHT	1	1:0.5	633.7	0.65	-4.83
ESAT 6:CHT	3	1:1	408.9 ± 51.02	0.54 ± 0.06	0.852 ± 1.1
ESAT6:CHT	3	1:2	356.3 ± 42.20	0.39 ± 0.05	3.23 ± 0.65
ESAT 6:CHT	3	1:4	735.6 ± 85.30	048 ± 0.07	0.982 ± 1.07
ESAT 6:CHT	1	1:6	793	0.77	0.557
ESAT 6:CHT	1	1:8	710	0.79	0..552
ESAT 6:TMC	3	1:1	309 ± 38.85	0.40 ± 0.05	2.94 ± 0.13
ESAT 6:TMC	3	1:2	470.3 ± 48.21	0.40 ± 0.015	5.03 ± 0.14
ESAT 6:TMC	3	1:4	440 ± 52.20	0.7 ± 0.08	1.12 ± 0.1

CHT: chitosan, TMC: trimethyl chitosan

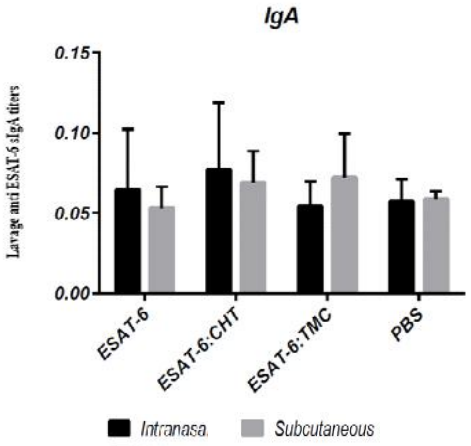

Fig. 3. Lavage anti ESAT-6 sIgA titers. Mice were immunized i.n. and s.c. with different formulations in days 0, 14 and 28. In the sixth week after the first immunization, mice nasal lavages were obtained and antibody titers were evaluated by an ELISA method.

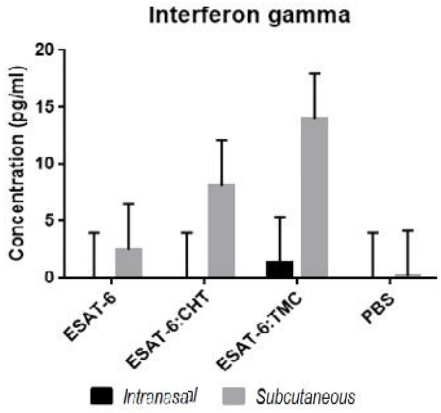

Fig. 4. IFN-γ concentration in supernatant of cultured spleen cells. Mice were immunized i.n. and s.c. with different formulations in days 0, 14 and 28. In the sixth week after the first immunization, mice splenocytes were cultured for 72 hours and IFN-γ concentration in supernatant was determined by an ELISA method.

DISCUSSION

Subunit vaccines are safe, naturally immunodominant and able to induce long term immunological memory. In recent years, many studies showed that Esat-6 antigen alone or in combination with the other MTb antigens can act as a promising inducer of protective immunity against TB [2, 13]. At the present study some humoral and cell mediated immune responses was seen against this antigen, however, higher responses was observed for nanoparticulate form of ESAT-6, i.e. CHT: ESAT-6 and TMC: ESAT-6 NPs. These are also several reports about the potentiation and adjustment of immune responses against an antigen after its encapsulation within or located onto their surface [14-17]. For optimal protection against TB, nasal administration of TB vaccine could induce effective humoral and cell mediated immune responses and help to protection against MTb [10, 11]. CHT and its derivatives like TMC are proven mucoadhesive polymers and they can also enhance the absorption across mucosa epithelium by opening tight junctions. Due to these features, CHT and TMC NPs have been widely used as nasal vaccine delivery system [18]. As Fig 2 shows, nasal administration of ESAT-6 antigen induced lower immune responses, compared with ESAT-6 encapsulated with NPs. It shows the immunoadjuvant potential of these NPs. This potential could be related to the mucoadhesion of these NPs and more residence time in the nasal cavity [19]. Microfold cells located in the nasal associated lymphoid tissue (NALT), could interact better with particulate form of antigen. Therefore, the higher immune responses observed with NPs could be attributed to their more uptakes with M cells [15, 16]. At the present study, the positive charge of the CHT NPs was lower than TMC NPs. In agreement with the other studies, it seems that CHT based NPs (i.e.

CHT and TMC) have different potentials in the stimulation of immune responses via intranasal vaccination [20]. After nasal delivery, TMC: ESAT-6 NPs induced higher IgG2a titers (P>0.05), IFN-γ (P<0.01) and IgG2a/IgG1 (P<0.001) ratios and lower secretory IgA (sIgA) (P>0.05) and IgG1 (P>0.05) titers than CHT: ESAT-6 NPs. It shows the superiority of TMC NP_s over CHT NPs in induction of Th1 immune responses via this route. The same results have been reported in other studies [12]. Formulations injected subcutaneously induced stronger immune responses compared with nasal administration. Mice after parenteral immunization with CHT: ESAT-6 levels of IgG1 and IgG total were higher as compared with TMC: ESAT-6 and ESAT-6 protein alone but differences were not statistically significant (P>0.05). Also, CHT: ESAT-6 and TMC: ESAT-6 NPs induced significantly the highest levels of IgG2a after subcutaneous immunization (P<0.001). These results are in agreement with other reports of Seferian *et al*, Martinez *et al* and Zaharoff *et al*. They showed that subcutaneous administration of CHT encapsulated with antigen significantly promoted antigen-specific antibody titers [18, 21, 22]. In this study, subcutaneous administration of TMC:ESAT-6 NPs induced significantly higher levels of IFN-γ, a representative of Th1 immune responses, compared with CHT:ESAT-6 NPs (P<0.01). Stimulation of sIgA antibodies against TB subunit antigens plays an important role in protection against mucosally transferred pathogens such as MTb [23]. As shown in Fig. 3, nasally administered formulations induced high sIgA titers. However, differences were not significant (Pe≥0.05). Mice received subcutaneous, TMC based formulations elicited greater levels of IFN-γ as compared to nasally immunized groups (P<0.01).

CONCLUSION

Both TMC: ESAT-6 and CHT: ESAT-6 NPs were stable up to 15 days, but afterwards they showed some signs of instability. Compared with CHT based NPs, TMC: ESAT-6 NPs showed more efficient in stimulation of Th1 immune responses. While regards to the importance of cell mediated immunity in protection against TB, and also the solubility in physiological pH, TMC NPs are more efficient adjuvant/delivery system for immunization against TB. Finally, according to the results this study, CHT NPs were better than TMC nanocarrier for nasal antigen delivery of TB.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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