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Research paper

Chitosan microspheres enhance the immunogenicity of an Ag85B-based fusion protein containing multiple T-cell epitopes of *Mycobacterium tuberculosis*

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Abstract

To develop novel delivery system for tuberculosis (TB) subunit vaccine, biodegradable chitosan microspheres were prepared and used to deliver a fusion protein, Ag85B–MPT64$_{190–198}$–Mtb8.4 (AMM for short), made from three *Mycobacterium tuberculosis* genes. AMM-loaded microspheres were first characterized for their morphology, size, zeta potential, loading efficiency, and in vitro release of AMM. C57BL/6 mice were immunized at weeks 1, 3 and 5 subcutaneously with AMM formulated in chitosan microspheres, in incomplete Freund's adjuvant (IFA), or in phosphate-buffered saline (PBS), respectively. Three weeks after the last immunization, humoral and cell-mediated immune responses were examined. It was shown that the microspheres bound AMM quite efficiently (loading efficiency: >99%). AMM-loaded chitosan microspheres were observed as aggregated shapes with the average particle size of 5.78 ± 0.65 μm and zeta potential of 32.77 ± 1.51 mV. In vitro release studies revealed that only small amount of antigen was released in 16 days. Following subcutaneous administration, splenocytes immunized with AMM in chitosan microspheres produced higher levels of IFN-γ compared to administration of AMM in PBS upon stimulation with Ag85B and synthetic peptide MPT64$_{190–198}$. The levels of Ag85B-specific IgG (H+L), IgG1 and IgG2a in sera of mice immunized with AMM in chitosan microspheres were also higher than those with AMM in PBS. These results indicate that chitosan microspheres when used as a carrier for fusion protein AMM could elicit strong humoral and cell-mediated immune responses.

Keywords: *Mycobacterium tuberculosis*; Subunit vaccine; Fusion protein; Chitosan microspheres; Adjuvant

1. Introduction

Tuberculosis (TB) remains a major health problem worldwide. One-third of the world population is infected with *Mycobacterium tuberculosis* (Mtb), and approximately 5–10% of those infected become sick or infectious at some time during their lifetime. Moreover, the percentage in whom progressive disease develops has been increasing markedly in recent decades due to the spread of HIV/AIDS and the emergence of multidrug-resistant TB. Although the Bacillus Calmette-Guérin (BCG) is widely administered in newborn children with good protective efficacy, its protective efficacy against adult pulmonary TB is poor. There is an urgent need to develop novel vaccines against TB, such as live mycobacterial and subunit vaccines [1–3].

Various protective antigens of Mtb have been identified, including Ag85B, MPT32, MPT64, ESAT-6, and Mtb 8.4, etc., which induce antigen-specific T cell mediated immunity when administered with suitable adjuvant [4–7].

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Furthermore, some T-cell epitopes have been identified, for example, the peptide $MPT64_{190–198}$ was confirmed to contain a $CD8^+$ T cell epitope [8]. These defined antigens and epitopes provide foundation for the development of subunit vaccines. However, the success of the subunit vaccines also needs advances in adjuvant development.

It is believed that an effective TB vaccine should induce both humoral and cell-mediated immune (CMI) responses [9,10]. However, the currently used adjuvant for human vaccines (based on aluminum salts) biases the immune response towards the Th2 pole, and is unable to induce potent T-cell responses of the Th1 type, which is characterized by the production of interferon-γ (IFN-γ) upon stimulation with antigen [11,12]. Biodegradable microparticles, composed of poly (lactic acid) (PLA) or poly (lactide-co-glycolide) (PLG), have been used previously to enhance the effectiveness of defined antigens from Mtb [13]. It is shown that particles of the appropriate size, ~1–10 μm in diameter, are readily phagocytosed by dendritic cells and other antigen presenting cells (APCs) and the antigen is presented on both MHC class I and II molecules; protein-microsphere vaccines are capable of producing cell-mediated and humoral type responses [14,15].

Over the last decade, another biocompatible and biodegradable material, chitosan and its nano- and microparticles were also investigated for delivery of hydrophilic macromolecules such as peptide protein, drugs and vaccines. Chitosan is a cationic polysaccharide obtained by the deacetylation of chitin, the major compound of exoskeletons in crustaceans. Chitosan and its microspheres have many advantages for vaccine delivery. First, chitosan could open the intercellular tight junctions and favour the paracellular transport of macromolecules. Second, chitosan nano- and microparticles are suitable for controlled drug and vaccine release. Third, chitosan nano- and microparticles are most efficiently taken up by phagocytotic cells. Thus chitosan and its derivatives could induce strong systemic and mucosal immune responses against antigens [16,17]. Association of vaccines to nanoparticles made of different molecular weight chitosan (23, 38, and 70 kDa) has shown to induce high and long-lasting immune responses [18]. Nasal chitosan influenza vaccine was both effective and protective in human, and nasal chitosan pertussis, diphtheria, and atrophic rhinitis vaccines also have good efficacy in animal models [17,19].

Besides enhancing the immune response by opening the intercellular junctions or stimulating the uptake by macrophages, chitosan may also stimulate the immune system as adjuvant [16]. Chitosan based adjuvant formulations were effective in sensitizing mice and guinea pigs for antigen specific DTH responses [20]. In addition, partially deacetylated chitin was effective in activation of macrophages after intraperitoneal administration in mice; and 70% deacetylated chitin was shown to possess adjuvant activity for induction of cell-mediated and humoral immunity [21]. Furthermore, chitosan stimulated the induction of cytokines like interleukin, interferon, and colony stimulating factor after intraperitoneal injection in mice. Only phagocytosable chitosan particles were able to induce significant interferon gamma levels and alveolar macrophage priming after intravenous administration [22]. These studies indicate that chitosan particles could stimulate macrophages, B and T lymphocytes. Therefore, chitosan nano- and microparticles used as immunological adjuvants or vaccine carriers are promising to induce both humoral and cell-mediated immunity. Recently, it was reported that pulmonary delivery of chitosan-DNA nanoparticles enhanced the immunogenicity of a DNA vaccine encoding HLA-A0201-restricted T-cell epitopes of *M. tuberculosis* [23]. However, there is still no report on the immune adjuvant effect of chitosan microspheres for TB protein vaccine.

In the present study, we prepared chitosan microspheres, and constructed a TB fusion polypeptide Ag85B–$MPT64_{190–198}$–Mtb8.4 (named AMM for short). Mice were immunized with the fusion protein delivered by the chitosan microspheres, and the immune responses were analyzed. The results show that the chitosan microspheres could deliver AMM to induce strong cellular and humoral immune responses against defined *M. tuberculosis* antigens.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice aged 6 weeks old were purchased from Slaccas Inc., (Shanghai, China). Animals were maintained in special pathogen-free conditions, and were used for experiments beginning 7–8 weeks of age. Mice received free access to food and water throughout the study.

2.2. Ag85B–$MPT64_{190–198}$–Mtb8.4 plasmid construction

The plasmid encoding Ag85B–$MPT64_{190–198}$–Mtb8.4 was generated by inserting the coding regions of $MPT64_{190–198}$–Mtb8.4 and the mature Ag85B polypeptide into the multiple cloning sites of the expression vector pET28a(+), respectively. Initially, the DNA sequences coding mature protein of Ag85B was generated by PCR amplification from *M. tuberculosis* H37Rv chromosomal DNA with the 5′-specific primer Ag85BF, and the 3′-specific primer Ag85BR (Table 1). The fragment was cloned into the *Eco*RI and *Sal*I site of pET28 to construct the plasmid pET28 Ag85B. Mtb8.4 gene was generated by PCR amplification from *M. tuberculosis* H37Rv genomic DNA with the Primer Mtb8.4F1 and Mtb8.4R. Then the coding region of $MPT64_{190–198}$–Mtb8.4 was generated by PCR amplification from PCR purified production of Mtb8.4 with the 5′-specific primer Mtb8.4F2 and the 3′-specific primer Mtb8.4R. This fragment was cloned into the unique site *Sal*I and *Hin*dIII of the previously constructed pET28 Ag85B plasmid. DNA sequences of the inserts were confirmed by sequencing. The final plasmid construct was transformed into the *Escherichia coli* strain BL21 for

Table 1
Primers used to engineer the fusion protein

Primers	Sequence
Ag85BF	CGAATTCTTCTCCCGGCCGGGGCT (*Eco*RI)
Ag85BR	ATAGTCGACGCCGGCGCCTAACGAACTCTGGAG (*Sal*I)
Mtb8.4F1	ACAGTCGACATGAGGCTGTCGTTGAC (*Sal*I)
Mtb8.4F2	ACAGTCGACTTCGCAGTCACGAACGACGGGGTGATTATGAGGCTGTCGTTGAC (*Sal*I)
Mtb8.4R	GCGAAGCTTTTAATAGTTGTTGCAGGAG (*Hin*dIII)

production of the fusion protein Ag85B–$MPT64_{190–198}$–Mtb8.4 (AMM).

2.3. Expression and purification of mycobacterial antigens

Recombinant Ag85B was produced as described previously [24]. $MPT64_{190–198}$ polypeptide was synthesized by Sangon company (China). Recombinant Rv3425 protein (as sham-control) was expressed and purified in our laboratory using pET32a (data not shown).

Escherichia coli BL21 expressing AMM was gown in culture flasks to an OD of ~0.5 at 600 nm before incubation with 1 mM isopropyl β-D-thiogalactopyanoside (IPTG). After incubation, growth was continued for 4 h at 37 °C. Cells were harvested by centrifugation at 12,000*g* for 10 min at 4 °C. Then cells were suspended in buffer B without urea (sodium phosphate buffer 0.1 M, Tris–Cl 0.01 M, pH 8.0) at 5 ml per gram wet weight, and sonicated on ice at 200–300 W for 30 min with 1 s cooling period between each 1 s bursting. The insoluble material containing the AMM aggregated in inclusion bodies was precipitated by centrifugation at 12,000*g* for 10 min at 4 °C, and AMM was solubilized and extracted overnight at 4 °C in buffer B (urea 8 M, sodium phosphate buffer 0.1 M, Tris–Cl 0.01 M, pH 8.0). AMM protein was subsequently purified by Ni–NTA His. Bind Resin-columns (Novagen) according to the manufacturer's instructions. Briefly, 1 ml of 50% Ni–NTA His Bind slurry was added to 4 ml lysate and mixed gently by shaking for 60 min at room temperature, the lysate-resin mixture was carefully loaded onto the column, which was washed with 2× 4 ml buffer C (urea 8 M, sodium phosphate buffer 0.1 M, Tris–Cl 0.01 M, pH 6.3). Finally, AMM was eluted with buffer D (urea 8 M, sodium phosphate buffer 0.1 M, Tris–Cl 0.01 M, pH 5.9) and buffer E (urea 8 M, sodium phosphate buffer 0.1 M, Tris–Cl 0.01 M, pH 4.5). Fractions containing AMM were identified by 12% SDS–PAGE and pooled. Finally, the pooled fractions were dialyzed against urea concentration gradient (6, 4, 2, 1, 0.5, and 0 M urea with 5 mM Tris–Cl, pH 7.9) for 12 h at each concentration at 4 °C. The concentration of the purified AMM was determined by the Lowry protein assay method.

2.4. Chitosan microsphere preparation

Chitosan (85% deacetylated) was purchased from Sigma–Aldrich (USA). Chitosan microspheres were prepared as described before with minor modification [19]. Briefly, a chitosan solution of 1.0% (w/v) was prepared in MilliQ water containing 2% (v/v) acetic acid and 1% (w/v) Tween 80. Then 5 ml of 10% (w/v) sodium sulfate was introduced dropwise to 100 ml chitosan solution under stirring and sonication (10 min, read-out 50, constant duty cycle). The microsphere suspension was subsequently centrifuged for 25 min (2750 rpm). The pellet was resuspended in MilliQ water to wash the microspheres and centrifuged again. After repeating this washing step, the pellet was placed at 4 °C for immediate use or freeze-dried as described before with little modification [19]. After the last centrifugation step, the pellet was immediately frozen in −80 °C overnight. Then the particles were freeze-dried without any cryoprotectant using a VirTus SENTRY™ freeze dryer. Before use as adjuvant, dried chitosan particles were suspended in MilliQ water for 2 days, followed by short time sonication (read-out 50) until fully resuspended.

The particle size and zeta potential of microspheres were determined using a ZetaPlus Zeta Potential Analyzer (Brookhaven Instrument Corporation, USA). The morphology of the microspheres was investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM pictures were obtained by air-drying AMM-loaded microspheres and sputter coating them with gold. Images were obtained using a TESCAN 5136 MM scanning electron microscope. For TEM photograph, the chitosan microspheres were stained with tungsten phosphate and imaged using a Philips CMI 120 TEM.

2.5. Loading of AMM protein

The AMM loading of microspheres was performed by incubating 0.6 ml of 1% (w/v) chitosan microsphere solution and 0.1 ml of 0.3% (w/v) AMM solution (Tris–Cl, pH 7.9) under shaking at 25 °C for 1 h, then incubated at 4 °C overnight. After incubation, the suspension was centrifuged (1400 rpm for 30 min) to remove free, unloaded AMM. The degree of microsphere loading was determined by quantifying the non-bound AMM in the supernatant with the Lowry protein assay method. Loading efficacy (LE) was determined as follows:

$$\mathrm{LE} = (\text{total of amount AMM} - \text{free AMM})/\text{total of amount AMM}$$

2.6. In vitro release studies

AMM release from chitosan microspheres was determined in trehalose solution (5% w/v), which was required to preserve the integrity of the microspheres following several centrifugation/resuspension cycles [18]. After loading, the microspheres were resuspended in trehalose solution to make a 1% (w/v) microparticle suspension. Samples of 1 ml were incubated in 1.5 ml microtubes at 37 °C. After 2 h, 1 d, 4 d, 8 d, 16 d, the tubes were centrifuged (8000 rpm for 30 min) and aliquots (1 ml) were withdrawn from the release medium and replaced by an equal volume of trehalose solution. The amount of AMM release was determined by an enzyme linked immunosorbent assay (ELISA) as described before with minor modifications [25]. Briefly, 96-well flat-bottomed NUNC-Immuno Plates (Inter Med, Denmark) were coated at 4 °C overnight with 100 μl of 10 μg/ml of rabbit anti-Ag85B serum in 50 mM carbonate buffer (pH 9.6). The plates were washed three times with 300 μl of phosphate-buffered saline (PBS) containing 0.05% Tween 20. Plates were blocked for 2 h at 37 °C with 200 μl/well 1% bovine serum albumin (BSA) in PBS. Plates were washed, and standard AMM protein with known concentration and samples were diluted at twofold steps in coated plates using PBS. The plates were held at 37 °C for 2 h followed by addition of mice anti-AMM serum (in PBS) at 37 °C for 1 h. Finally, peroxidase-conjugated goat anti-mouse IgG(H+L) (Jakson ImmunoResearch Laboratories, Inc.) at a 1:10,000 dilution was added for 1 h at 37 °C. Plates were detected with *o*-phenylenediamine (OPD) buffer (containing 0.03% hydrogen peroxide, pH 5.0), and the reaction was stopped by adding 2 N H_2SO_4. The OD was determined at 492 nm. The concentration of antigenic AMM in release medium samples was determined against standard AMM.

2.7. Mouse immunization

Previous study showed that among the mice immunized with three different concentrations (75, 37.5, 18.75 μg) of Ag85B in three doses at weekly intervals, the T-cell proliferation and antibody titer increased with increasing doses of antigen and were maximum at the third week p. im. in animals with 75 μg concentration of antigen. The protective efficacy was also the highest in animals immunized with 75 μg concentration of Ag85B protein and it was lower with decreased dosage of the antigen [5]. So in our experiment we applied high dose of the antigen: 100 μg protein in incomplete Freund's adjuvant (IFA). And considering the delayed release of antigen in chitosan particles, the protein in the microspheres was reduced to 50 μg.

C57BL/6 mice were immunized subcutaneously with 100 μg of Ag85B protein in IFA (Sigma), 50 μg of AMM polyprotein delivered by Chitosan microspheres (equivalent to 1.0 mg particles), and 100 μg of AMM protein formulated in IFA or in PBS, respectively. The animals were immunized with three doses at weeks 1, 3 and 5. Control animals were sham-immunized with PBS, Chitosan microspheres, and IFA only. Three weeks after the last injection serum was collected, and mice were sacrificed to conduct the analysis of immune responses.

2.8. ELISA for anti-Ag85B serum IgG(H+L), IgG1 and IgG2a

Serum samples were taken from all animals, and Ag-specific ELISAs were performed for the identification of specific anti-Ag85B IgG(H+L), IgG1 and IgG2a. The ELISA was performed as above with some modifications: Microtiter plates were coated with 100 μl/well of Ag85B at 5 μg/ml in PBS; serum samples were diluted to 1:100 with PBS and applied to plates in twofold serial dilutions; peroxidase-conjugated goat anti-mouse IgG(H+L) (Jakson ImmunoResearch Laboratories, Inc.) at a 1:10,000 dilution, peroxidase-conjugated rabbit anti-mouse IgG1 or IgG2a (Zymed Laboratories Inc.) at a 1:1000 dilution was added, respectively.

2.9. ELISPOT for IFN-γ from spleen cell cultures

Spleens were aseptically removed and gently ground through a 70 μm cell strainer, then single-cell suspensions were prepared with Lympholyte-M density gradient centrifugation (Cedar Lane Lab). IFN-γ ELISPOT kits (U-Cytech BV, Utrecht, The Netherlands) were used according to the instruction manual. In brief, 96-well transparent polystyrene plates were coated with 50 μl anti-IFN-γmAb overnight at 4 °C. The plates were then washed five times with PBS containing 0.05% Tween 20 (PBST), and then blocked with 200 μl Blocking solution B overnight at 4 °C. Blocking solution B was discarded from the plates, and freshly isolated spleen cells were plated in duplicate at 5×10^5 cell per well in 100 μl of RPMI 1640 supplemented with penicillin, streptomycin, and 10% newborn calf serum (GIBICO, New Zealand) and stimulated with Ag85B 2 μg/ml, or $MPT64_{190–198}$ 0.2 μg/ml for 48 h at 37 °C, 5% CO_2. The cells were then removed, and 200 μl/well ice-cold deionized water was added to lyse the remaining cells. The plates were incubated on ice for 15 min, after which they were washed 10 times with PBST. Next, biotinylated detector Ab solution was added and the plates were incubated for 1 h at 37 °C. The plates were washed 10 times with PBST, after which 50 μl/well ϕ-labeled anti-biotin antibodies (GABA) were added. The plates were again incubated for 1 h at 37 °C and washed five times with PBST. A total of 30 μl/well activator mix was then added, and the plates were developed for 30 min. Once these sites or black spots could be seen in the wells under an inverted microscope, the wells were washed with distilled water to stop development. The plates were then air-dried, and spots were counted.

2.10. Statistical analysis

All experiments were done in triplicate under the same condition. The results were expressed as means ± standard deviation (SD), and the significance of differences among the groups were determined by *t*-test. Probability values ($p < 0.05$) were considered as significant.

3. Results and discussion

3.1. Construction of AMM

Since Ag85B, $MPT64_{190–198}$, and Mtb8.4 were confirmed to be the effective protective antigens (or antigen epitopes) [5–8], they were fused together to construct a new subunit vaccine. The DNA sequences of recombinant fusion protein, Ag85B–$MPT64_{190–198}$–Mtb8.4, were confirmed by sequencing, and were translated into protein (Fig. 1a). AMM was produced in *E. coli* as inclusion bodies, which were subsequently dissolved, and purified with Ni–NTA His. Bind affinity chromatography column. Finally, the AMM protein was purified to homogeneity and analyzed by SDS–PAGE (Fig. 1b).

3.2. In vitro characterization of chitosan microspheres

Chitosan microspheres were prepared by a precipitation/coacervation technique using chitosan and sodium sulfate as precipitant agent. Then AMM protein in Tris–Cl buffer (5 mmol/L, pH 7.9) was mixed with chitosan microspheres. Fig. 2 shows SEM micrographs of AMM-loaded chitosan microspheres. The micrographs of SEM and TEM (data not shown) showed that the single AMM-loaded chitosan particle was spherical with the size of about 100–300 nm, and several nanoparticles aggregated together into bigger ones. Then the particle size was determined by laser light setting. The result showed that The particle size of chitosan particles themselves and AMM-loaded chitosan particles was 4.59 ± 0.41, and 5.78 ± 0.65 μm, respectively. The zeta potential of the chitosan microspheres resuspended in MilliQ water was measured to be $+36.62 \pm 1.20$ mV, which reduced to $+32.77 \pm 1.51$ mV after loading of AMM into the chitosan microspheres.

Microspheres less than 10 μm size are reportedly taken up by the APCs including macrophages and dendritic cells and thus considered to be most suitable for optimum adjuvant effect [13–16,26]. The size of AMM-loaded chitosan particles we prepared was 5.78 ± 0.65 μm, so that the particles could be efficiently taken up by APCs.

The loading efficiency of AMM in the microspheres was over 99% as determined by the Lowry protein assay method. To investigate the release of AMM from loaded chitosan microspheres, these microspheres were resuspended in trehalose solution and incubated for 2 h to 16 days at 37 °C. It was shown that little AMM was spontaneously released over a period of 16 days in vitro (Table 2). Since

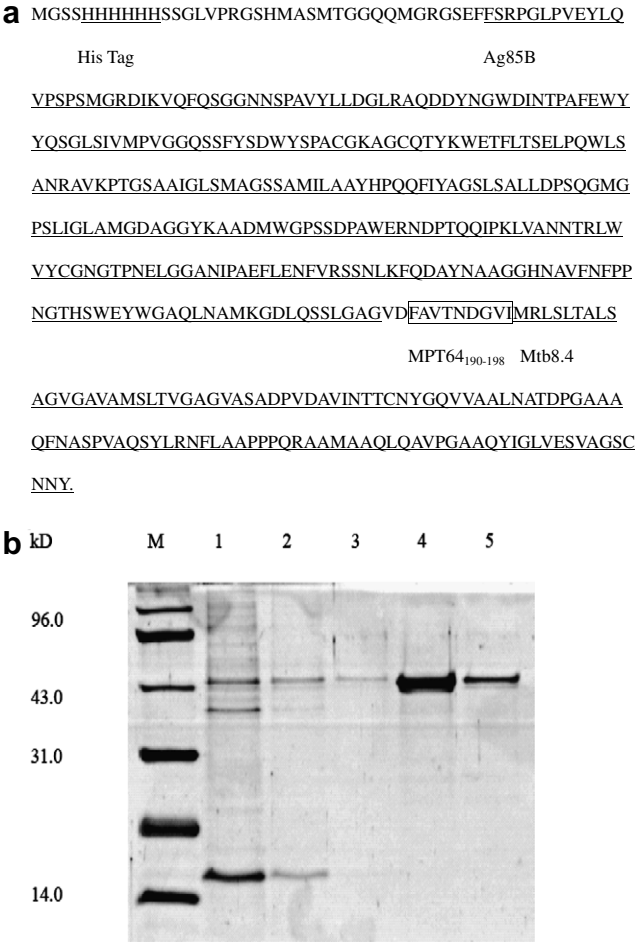

Fig. 1. (a) The translation product of AMM (Ag85B–$MPT64_{190–198}$–Mtb8.4) with the molecular weight of 46.7 kDa. (b) Purification of AMM produced in *E. coli*. The *E. coli* strain BL21 transformed with the expression vector harboring the AMM was grown and induced with IPTG. AMM was purified from inclusion bodies by Ni–NTA His Bind column. The figure shows a Coomassie blue-stained 12% SDS–PAGE of the purified protein. M, protein molecular weight marker; 1–3, washed with buffer C with pH 6.3; 4 and 5, AMM was eluted with buffers D and E with pH 5.9 and 4.5, respectively.

there was little AMM release, all vaccine remained well associated with the microspheres. Therefore, it is most likely that AMM is only released after biodegradation of the particles, which may occur by lysozymes in the cell [19,27].

3.3. The immunity of fusion protein AMM

In the present study, we constructed a fusion polypeptide AMM containing several major protective antigens (peptide) including Ag85B, $MPT64_{190–198}$, and Mtb8.4 [5–8]. To compare antigen-specific immune responses primed by single and fusion protein immunization, humoral immune responses were examined in mice immunized with Ag85B and AMM formulated in IFA. The antibody titers against Ag85B were determined in sera harvested from immunized mice three weeks after the last immuniza-

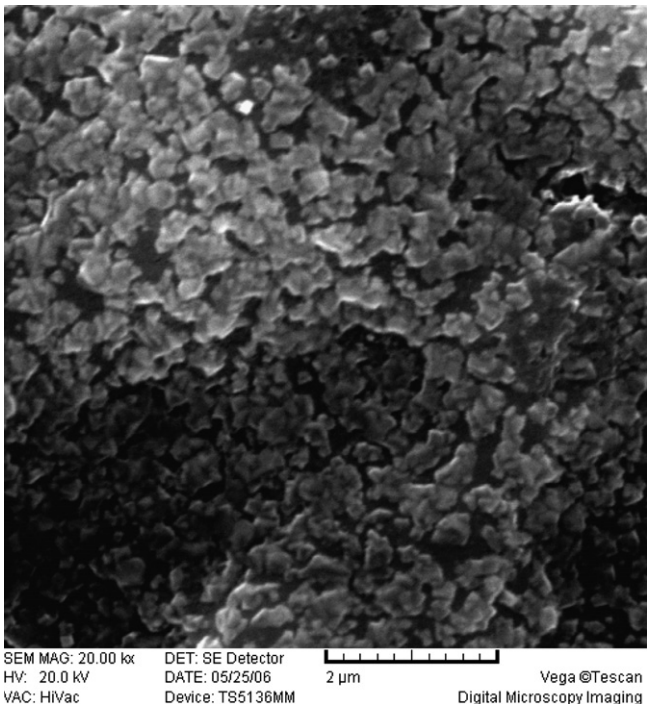

Fig. 2. Scanning electron micrograph of AMM-loaded chitosan microspheres.

tion. Serum from animals immunized with AMM contained significantly higher levels of Ag85B-specific IgG(H+L), IgG1, and IgG2a than serum from mice immunized with single Ag85B ($p < 0.05$). Serum from animals receiving IFA alone was negative (Fig. 3). The results indicate that the humoral immune response against Ag85B induced by the fusion protein AMM was stronger than that induced by single Ag85B.

Because of the complexity of the genetic restriction imposed by MHC molecules, the fusion protein AMM, containing several protective T-cell epitopes, has the advantage of ensuring a broad coverage of a genetically heterogeneous population [28]. Two components of AMM, Ag85B and $MPT64_{190–198}$, were used to examine the cell-mediated immune responses after vaccinated with AMM formulated in IFA. The results showed that both of them could be effectively recognized and induce high levels of IFN-γ secretion (Fig. 4). In summary, the subunit vaccine based on AMM did generate strong immune responses in mice. Therefore, AMM was a good candidate of subunit vaccine.

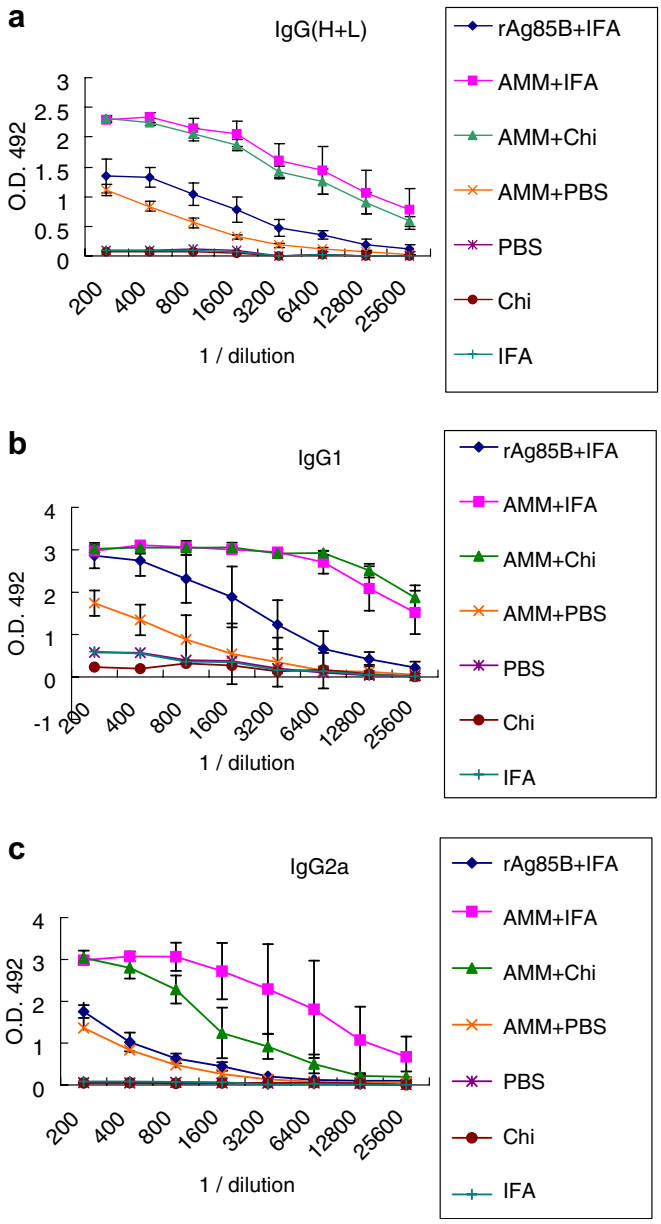

Fig. 3. Serological responses to Ag85B in immunized mice. C57BL/6 mice were immunized subcutaneously with 50 μg of AMM delivered by chitosan microspheres, 100 μg of AMM protein formulated in IFA (sigma), or 100 μg of Ag85B protein in IFA at weeks 1,3 and 5. Control animals were immunized with PBS, chitosan microspheres alone, or IFA without protein. Serum samples were collected 3 wk after the last injection and analyzed by ELISA for the presence of anti-Ag85B IgG(H+L) (a), IgG1 (b) and IgG2a (c). Each point represents the mean of data from three individual mice.

Table 2
AMM release from chitosan particles over time in vitro

	2 h	1 day	4 days	8 days	16 days
Amount of AMM (ng)	12.60 ± 3.54	0	10.47 ± 2.24	14.37 ± 6.86	11.67 ± 1.73
Distributive percentage (%)	0.002	0	0.002	0.002	0.002

Total amount of 600 μg AMM protein was associated with chitosan particles in every sample. Distributive percentage of AMM released in vitro with respect to the total amount of protein is almost zero.

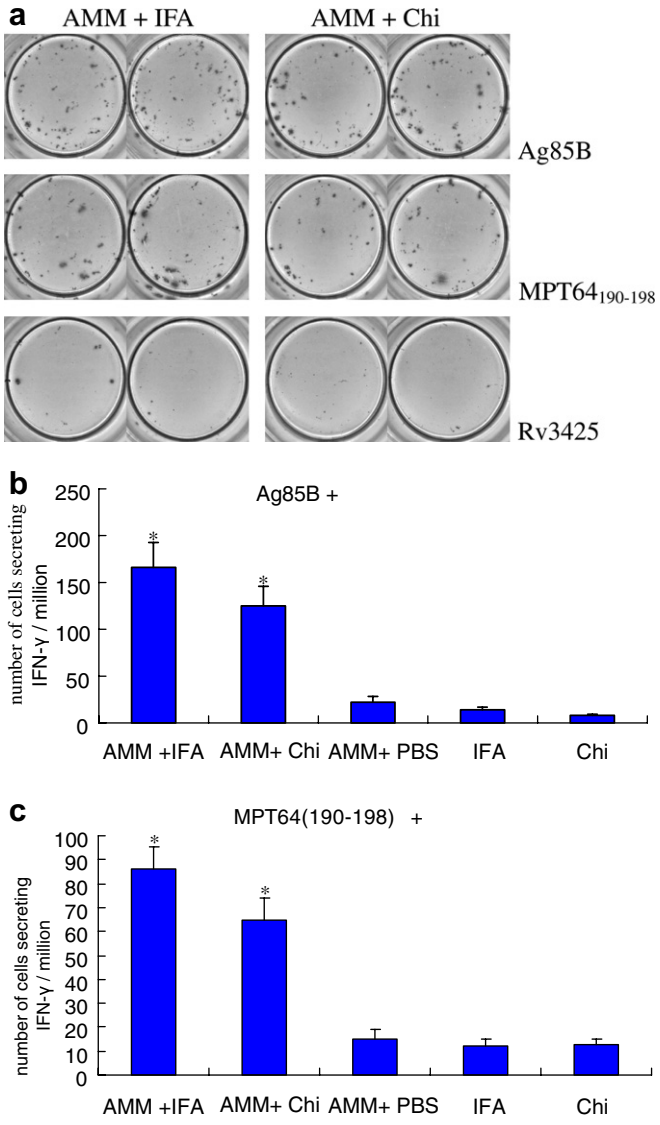

Fig. 4. IFN-γ secretion in splenocytes following stimulation with Ag85B and $MPT64_{190–198}$, respectively. Mice were immunized with AMM (100 μg/ml) in IFA, AMM (50 μg/ml) in chitosan microspheres (Chi), and AMM (50 μg/ml) in PBS, respectively. IFA and chitosan microspheres were used as sham control. Freshly isolated spleen cells were plated in duplicate at 5×10^5 cell per well in 96 spot and incubated with Ag85B (2 μg/ml) or $MPT64_{190–198}$ (0.2 μg/ml) for 48 h at 37 °C, 5% CO_2. (a) Elispot analysis of mice immunized with AMM in IFA or chitosan microspheres. Recombinant Rv3425 protein (10 μg/ml) as a sham control. (b) The number of cells secreting IFN-γ per 1×10^6 cells following stimulation with Ag85B. (c) The IFN-γsecreting cells per 1×10^6 cells with stimulation of $MPT64_{190–198}$. Data shown are from pooled cells from more than three mice as means ± SD. *$P < 0.05$ vs. AMM in PBS.

3.4. Chitosan microspheres induced effective humoral and cell-mediated immune responses

Although IFA has proven to induce Th1 type immune response, for safety reasons, it could not be applied in clinic. Therefore, it is a big challenge to find a safe adjuvant to replace IFA. As a novel mucosal delivery system for DNA and subunit vaccines, biodegradable chitosan microparticle was effective in inducing cell-mediated and humoral immune responses [16–19]. In this study, chitosan microspheres were used to deliver AMM protein. After mice were immunized subcutaneously with AMM in different adjuvants, humoral and cell-mediated immune responses were examined.

In sera of mice immunized with AMM delivered by chitosan microspheres, the levels of Ag85B-specific IgG (H+L) and IgG1 were almost as high as in mice immunized with AMM formulated in IFA ($p < 0.05$); although the level of Ag85B-specific IgG2a was slightly lower than in mice immunized with AMM in IFA but was higher than in mice immunized with AMM in PBS ($p < 0.05$) (Fig. 3).

The IgG2a isotype is associated with a Th1-type cytokine response during which IFN-γ is produced. This corresponds to the finding that splenocytes from mice vaccinated with AMM delivered by chitosan microspheres or formulated in IFA produced IFN-γ when stimulated in vitro with Ag85B and synthesized $MPT64_{190–198}$ (Fig. 4).

Production of immunoglobulin isotypes is associated with different T-helper responses. Th1 cells secrete IFN-γ and interleukin-2 (IL-2), promoting the production of IgG2a. Th2 cells secrete IL-4, IL-5 and IL-10, promoting the production of IgG1 [29]. The antibody response of mice immunized with AMM delivered by chitosan microspheres had both IgG1 and IgG2a antibodies present. The anti-Ag85B IgG1 antibody titers elicited by AMM in chitosan microspheres were similar to those elicited using IFA. The IgG2a titers from mice immunized with AMM-chitosan microspheres were not as strong as those from mice immunized with AMM in IFA but were obviously stronger than in PBS. In addition, little IL-4 secretion was detected in splenocytes from mice immunized with AMM in chitosan microspheres and in IFA by ELISA (data not shown), while IFN-γ production was detected. The results indicated that AMM vaccine delivered by chitosan microspheres was able to induce Th1 type cell-mediated immune response. Furthermore, because $MPT64_{190–198}$, a $CD8^+$ T-cell epitope, could stimulate the splenocytes to produce IFN-γ, $CD8^+$ T-cell responses were also induced [8,12,16,30].

Chitosan microspheres with appropriate size, ~1–10 μm, are readily taken up by APCs [13–16,26]. Antigen is released within the cell when chitosan is degraded by the lysozymes [19,27]. Subsequently, the antigen was presented through MHC classI and MHC class II molecules to $CD8^+$ T cells (CTL) and $CD4^+$ T cells (Th cells), respectively [9,12]. In addition, chitosan microspheres could act as immune potentiators to directly activate innate immune cells, which provide the pro-inflammatory context for antigen recognition. For example, it could enhance the production of IL-6, IL-12 and IFN-γ, which is attributable to the production of Th1 type immunity [16,22].

Safety is the main hurdle to the development of new and improved adjuvants. Many adjuvants for TB subunit vaccine had been extensively evaluated both preclinically and clinically, however, few have been successfully licensed for use in clinic because they are either ineffective at

stimulating CMI responses or too toxic for human use [3,30]. Chitosan has the huge advantage of being safe because it is biocompatible with human body and does not cause allergic reactions and rejection. It breaks down slowly to harmless products (amino sugars), which are completely absorbed by living tissues [31]. Biodegradable chitosan has been used for a variety of biomedical purposes including preparation of a cholesterol-lowering agent [32].

There may be some shortcomings for chitosan microspheres as an adjuvant or delivery system for TB subunit vaccine. Chitosan microspheres induced a mixed humoral and cell-mediated immune response rather than mainly Th1 type responses. In order to strengthen Th1 type response, some kind of immune potentiators and immune mediators such as IL-6 and IL-12 could be added to modify the effect of chitosan microspheres [33]. Future studies aimed at improving the immune activity of chitosan microspheres and evaluating their use as vaccine carrier in the disease protection study in animal models are needed.

4. Conclusions

The results show that chitosan microspheres could bind fusion protein AMM effectively and introduce AMM to elicit strong humoral and cell-mediated immune responses in mice. These findings suggest that biodegradable chitosan microspheres could be developed into a promising novel subcutaneous delivery system for the TB subunit vaccine.

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