Immunogenicity and protective efficacy study using combination of four tuberculosis DNA vaccines

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Abstract Immune response and protective efficacy for the combination of four tuberculosis DNA vaccines were evaluated in this study. We obtained 1∶200 antibody titers against Ag85B 21d after mice were vaccinated for the first time by four recombinant eukaryotic expression vectors containing coding sequences for Ag85B, MPT-64, MPT-63 and ESAT-6. The titers of Ag85B were elevated to 1∶102400 after the second injection and decreased to 1∶12800 after the third injection. Antibody titers for MPT-64 and MPT-63 reached 1∶25600 21 d after the first vaccination, and were then decreased following the second and third injections. No antigen-specific antibody titer against ESAT-6 was detected in sera harvested from immunized mice at any time. These DNA vaccines evoked specific IFN-γ responses in the spleens of vaccinated mice as well. When challenged with *M. tuberculosis* H37Rv, we found that the lungs of the vaccinated mice produced 99.8% less bacterial counts than that of the empty-vector control group and the bacterial counts were also significantly less than that of the BCG group. Histopathological analyses showed that the lungs of vaccinated mice produced no obvious caseation while over 50%—70% of the pulmonary parenchyma tissue produced central caseation in the vector control group. Our results indicated that the combination of four tuberculosis DNA vaccines may generate high levels of immune responses and result in better animal protection.

Keywords: ***Mycobacterium tuberculosis*, secreted proteins, combination DNA vaccines, immunogenicity, protective efficacy.**

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Tuberculosis is an ancient scourge of mankind. According to statistics, there are more than 10 million new cases of tuberculosis each year and the annual death toll for tuberculosis exceeds three millions. The current available BCG is of questionable efficacy, and its protection ranges from 0 to 85%. Therefore, developing a safe and effective vaccine against this scourge is very important. Previous studies have shown that the secreted proteins of *Mycobacterium tuberculosis* (M. TB) can induce protective responses by stimulating mycobacterium-specific $CD4^+$T lymphocytes as well as the antigen-specific cytotoxic $CD8^+$ T cell productions.

During the course of TB infection, proteins secreted by mycobacteria are recognized by lymphocytes of the patients. Ag85B complex is secreted by most mycobacterial species and it possesses mycolyltransferase enzyme activity that is needed for the attachment of mycolic acids to the cell wall and is also involved in the course of formative disease development. It is by far the most abundant extracellular protein in *M. tuberculosis* culture and produces the strongest antigen-specific responses in a number of mice immunization experiments[1—6]. MPT-64 is secreted by *M. tuberculosis*, by the virulent *M. bovis* and by a few *M. bovis* bacilli Calmette-Guerin (BCG) species. It is recognized by sera prepared from human TB patients and is capable of eliciting cytotoxic T-cell response (CTL)[7—9]. ESAT-6 is an early-secreted protein of lower molecular weight present in the culture filtrates of *M. tuberculosis* and *M. bovis*. It is absent in most BCG species. Previous studies have demonstrated that ESAT-6 may be a key target molecule in T-cell response during *M. tuberculosis* infection since it stimulated the mice infected with *M. tuberculosis* to release IFN-γ[10—12]. MPT-63 is specifically expressed in pathogenic strains of *M. tuberculosis* species and is able to evoke humoral immune responses in guinea pigs infected with the pathogen. Therefore, genes encoding these proteins are among the first candidates for DNA vaccines[13].

Recent studies have demonstrated that the combination of tuberculosis DNA vaccines is a promising new approach. Mice immunized with a multivalent combination DNA vaccine encoding two antigens of malaria produced strong resistance to the pathogen[14]. Morris and colleagues found that multivalent DNA vaccines evoked stronger immune responses and resulted in a better protection compared to BCG vaccine[15]. The purpose of our study is to develop combination tuberculosis DNA vaccines using genes encoding Ag85B, MPT-64, MPT-63 and ESAT-6 antigens, and to study their immune responses as well as their ability to protect mice from being infected with M. TB. We think that our study will lay a theoretical foundation for DNA vaccine development both in humans and animals.

1 Materials and methods

1.1 Materials

The eukaryotic expression vector pJW4303 was kindly provided by Prof. Mullins of Stanford University. COS7 cells were obtained from Shanghai Institute of Immunology, and the monoclonal antibodies of L24B4 and HYB76-8 were from Statens Seruminstitut Copenhagen (Denmark). *M. tuberculosis* H37Rv strain was produced by the Center of Tuberculosis Research, 309 Hospital of PLA, Beijing. Prokaryotic expression vector pET22B was from our own laboratory. Pathogen-free C57BL/6 female mice were obtained from the Animal Center at the Academy of Military Medical Sciences of PLA and were maintained with commercial mouse chow and water in the Center of Tuberculosis Research, 309 Hospital of PLA. All restriction enzymes were purchased from Promega, and QIAquick Gel Extraction Kit was purchased from QIAGene Company. All primers were synthesized from GibcoBRL.

1.2 Methods

1.2.1 Construction of recombinant eukaryotic expression vectors containing different target genes. Each PCR product was digested with *Nhe* I and *Bam*H I before being ligated to the eukaryotic expression vector pJW4303 (fig. 1) and subsequently transformed into *E. coli* DH5α cells. All the four genes encoding Ag85B, ESAT-6, MPT-64 and MPT-63 were fused respectively to TPA signal sequences. Plasmid DNA was digested with restriction endonucleases to determine the insertion size and was further verified by commercial DNA sequencing. Recombinant vectors were then transformed into *E. coli* Top10 strain. Colonies containing the vector were selected on LB-ampicillin (100 μg/mL) plates. Plasmid DNA was prepared and purified using the Qiagen plasmid Maxi and Mega kit. DNA was quantified by using a light/UV spectrometer and was diluted in saline solution to final concentrations of 1—2 μg/μL.

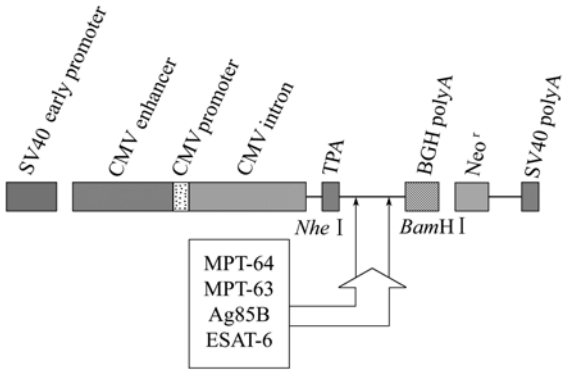

Fig. 1. Construction of the recombination eukaryotic expression vector containing Ag85B, MPT -64, MPT -63 and ESAT-6 coding sequences. CMV, Cytomegalovirus early promoter; TPA, tissue plasminogen activator signal sequences; Neo^r, A neomycin resistance cassette; BGH poly A and SV40 poly A, polyadenylation site using the BGH polyA signal or the SV40 poly A signal; double arrow, foreign gene insertion site.

1.2.2 Expression of four recombinant proteins from M.TB. ESAT-6, MPT-64 and MPT-63 antigens were expressed from the pGEX-4T-1 recombinant plasmid and Ag85B antigens were expressed from the pET22b recombinant plasmid. Bacterial lysates prepared from 1 mmol/L IPTG induced cultures were loaded directly onto SDS-PAGE for analysis.

1.2.3 Co-immunization of mice with the DNA vaccines. Ten C57BL/7 mice were immunized three times at 3-week intervals by the combination of four tuberculosis DNA vaccines. One hundred micrograms each of the plasmid DNA was injected intramuscularly into the tibialis anterior muscle of each hind leg. Control mice were immunized with three hundred micrograms of the parental vector pJW4303 or were vaccinated with 10^6 CFU of BCG Pasteur injected subcutaneously.

1.2.4 Analysis of specific antibodies using the indirect enzyme-linked immunosorbent assay (ELISA). Vaccinated mice were bled 21 days after the first, second and third DNA injections from the orbits, and sera collected from 5 mice were pooled. All sera were diluted in 2-fold sequentially starting from 1∶25 in PBS/ 0.05% Tween 20. Briefly, goat anti-mouse IgG HRP-conjugated antibody (1∶2000), IgG1 (1∶800) and IgG2a (1∶500) were used. The end point titer was defined as the highest dilution of serum that gave an absorbance value exceeding an optical density of 0.05 and was 2-fold greater than that of the matched dilution of normal mouse sera as described previously[16].

1.2.5 Determination of IFN-γ levels. IFN-γ induction and detection were performed as previously described[16]. The end point titer was defined as the highest dilution of supernatants that gave an absorbance value that exceeded an optical density of 0.05 and was 2-fold greater than that of the matched dilution of normal mouse spleen cell culture supernatants. The amount of IFN-γ was determined by using a standard dilution curve.

1.2.6 Evaluation of the protective efficacy of immunized mice. Six weeks after the last injection of the combination of four DNA vaccines, immunized mice were challenged with 10^6 CFU of *M. tuberculosis* H37Rv via a lateral tail vein. Vaccinated mice were sacrificed 7 weeks after the challenge. Tenfold serial dilutions of lung homogenates were plated on Lowenstein-Jensen medium and colonies were counted visually after four weeks of culture.

1.2.7 Histopathologic analyses. Tissue sections were fixed in 10% neutral buffered formalin for routine microscopic observations. All sections were stained with hematoxylin and eosin. The following parameters were used for assessing tissue sections: severity (degree of parenchymal involvement), size of typical granulomas, amount of caseous necrosis, relative number of neutrophils and lymphocytes and demarcations of the granulomas as compared to surrounding tissue.

1.2.8 Statistical analysis. Data were evaluated using the student *T*-test.

2 Results

2.1 Expression of four recombinant proteins

Upon IPTG (1 mmol/L) induction, crude cell extracts were prepared and separated by SDS-PAGE. The recombinant plasmids generated new polypeptides with apparent MW of 30, 50, 43 and 33 kDa corresponding to the naked Ag85B and to MPT-64, MPT-63, ESAT-6 peptides attached to the GST sequence respectively (data not shown).

2.2 Immunized mice showed high specific antibody titers

Twenty-one days after the first, second and third vaccinations of mice with the combination DNA vaccines, specific antibody titer for Ag85B was 1∶200, 1∶102400 and 1∶12800 respectively. The antibody titers for both MPT-64 and MPF63 were 1∶25600 when tested 21 d after the first vaccination. However, the titers decreased to 1∶400 and 1∶200 21 d after the third injection. No antigen-specific antibody against ESAT-6 was detected in the mice over the whole period of immunization (table 1). In both control groups, one injected with the empty vector DNA

Table 1 Humoral responses after the application of tuberculosis DNA vaccines

Antigens	End point titer		
	after 1st injection	after 2nd injection	after 3rd injection
Ag85B	1∶200	1∶102400	1∶12800
MPT-64	1∶25600	1∶12800	1∶400
MPT-63	1∶25600	1∶50	1∶200
ESAT-6	–	–	–

Four antigens induced the same antibody titers in two independent experiments.

and the other receiving no DNA injection, we found no specific antibodies against any one of the 4 proteins (data not shown).

2.3 IgG1and IgG2a analysis in immunized mice

Since the relative IgG2a and IgG1 antibody levels are a marker for the overall T-cell responses, we also determined these values using sera collected from vaccinated mice by the ELISA technique. After the third vaccination with the combination DNA vaccines, relatively high IgG1 antibody titers against Ag85B, MPT-64 and MPT-63 were recorded. Only very low IgG2 response against Ag85B was detected, and no antigen-specific IgG2 antibody against the other three antigens was detected (table 2). These antibody isotype results clearly suggest that the T-cell phenotype induced by DNA vaccine may be determined by the characteristics of the specific antigen.

Table 2 Determination of IgG1 and IgG2a production 21 d after the third injection of the combination DNA vaccines

Antigens	IgG1	IgG2a
Ag85B	1∶6400	1∶25
MPT-64	1∶100	–
MPT-63	1∶200	–
ESAT-6	–	–

The same results were obtained in two independent experiments.

2.4 Analysis of interferon (IFN-γ) production in vaccinated mice

Application of purified Ag85B, MPT-64, MPT-63 and ESAT-6 polypeptide to cultured spleen cells obtained from three C57BL/6 mice vaccinated with the combination DNA vaccines 3 weeks after the third injection was able to generate robust IFN-γ responses (fig. 2). Sandwich ELISA assay indicated that different amounts of antigen-specific IFN-γ were produced after the stimulation of various antigens. Mice immunized with combination DNA vaccines produced 6.9 + 0.2 ng/mL, 1.0 + 0.1 ng/mL, 1.2 + 0.07 ng/mL and 0.8 + 0.01 ng/mL IFN-γ after Ag85B, MPT-64, MPT-63 and ESAT-6 stimulation respectively. BCG-vaccinated mice produced 2.4 + 0.4 ng/mL IFN-γ and pJW4303 vector-DNA-vaccinated mice produced less than 0.1 ng/mL IFN-γ (fig. 2).

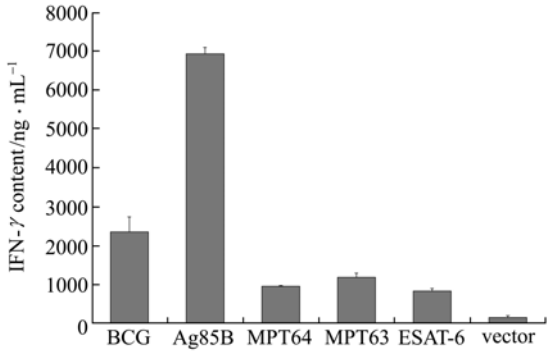

Fig. 2. Analysis of the level of specific IFN-γ in sera prepared from differently treated mice.

2.5 Analysis of protective immunity by the DNA vaccines

Protective responses were evaluated 7 weeks after challenging the three-time vaccinated mice with *M. tuberculosis* strain H37Rv. Lungs of mice vaccinated with all 4 expression vectors showed less than 0.2% of the bacteria burden when compared to lungs that were vaccinated with empty vectors (table 3). Mice that received combination DNA vaccines performed significantly better than those receiving BCG both in terms of bacterial burden per lung and in terms of the

calculated protective efficacy (table 3).

Table 3 Induction of protective immunity as evidenced in bacterial burden per lung and in the resultant protective efficacy

Vaccines	Bacterial burden (CFUs/ lung)	Protective efficacy[a)]
Vector DNA	（8.0±1.9） 10^9	–
Combination DNA vaccines	（1.4±0.5） 10^{7} [b)]	2.63
BCG	（2.0±1.3） 10^7	1.89

a) Calculated protection efficacy as described by Morris et al.[15]. b) Statistical significance: $P < 0.05$.

2.6 Histopathologic analyses of the vector control and vaccinated mice

Lung sections were evaluated 7 weeks after intravenous challenge of vaccinated and control mice. The microscopic analysis showed that in mice injected with the empty vector DNA, lungs became largely caseated with about 50%—70% of the pulmonary parenchyma cells involved. There was also extensive lymphocytes infiltration (plate I-1, 2). In mice that received the combination DNA vaccines, the lung tissue was normal and the alveolar tissue appeared to be intact. Apart from a few scattered lymphocytes, almost no granulomas were observed in these sections (plate I-5, 6).

For mice that received BCG vaccination, lung caseation was not apparent, and the granulomas were small and of limited extent (plate I-3, 4). However, lungs of these mice had significantly more lymphocytic infiltrations than that of mice injected with the combination DNA vaccines (plate I).

3 Discussions

The current study demonstrated that the combination of four tuberculosis DNA vaccines was able to elicit humoral responses against M.TB and different antigens might induce various responses via different mechanisms. The specific antibody titers elicited by Ag85B reached 1∶102400 after the second injection and decreased to 1∶12800 after the third injection. Both MPT-64 and MPT-63 induced high specific antibody titer (1∶25600) after the first vaccination and were decreased quickly after the second injection (table 1). No specific antibody against ESAT-6 was detected throughout the whole experimental period (table 1). The specific antibody titer in response to purified Ag85B in our combination DNA vaccines was about a 100-fold higher than that of Ag85B single DNA vaccine as reported by Fan et al.[18] and was equivalent to that reported by You et al. using single DNA vaccine[19] and by Kamath et al. using triplevalent DNA vaccine (Ag85B, MPT-64 and ESAT-6)[20]. These results indicated that Ag85B may be one of the best candidate protective antigens in the future studies of combination DNA vaccines.

Since IFN-γ plays a very important role in the induction of protective responses, the production of IFN-γ is often used as a key indicator for evaluating the effectiveness of the vaccines[21]. In the present study, we detected robust antigen-specific IFN-γ responses in splenocytes of mice vaccinated with the combination DNA vaccines (fig. 2). The Ag85B-specific IFN-γ content was about twofold higher than that of Tanghe et al.[22] and was fourfold higher than that of the ten tuberculo-

sis DNA vaccines tested by Delogu et al.[23]. These results suggested that the combination of four tuberculosis DNA vaccines was able to induce sufficient immune responses in properly vaccinated mice.

In a different study, the scientist found that in mice immunized either with combination DNA vaccines encoding MPT-64, MPT-63, ESAT-6 and Kat G or with ESAT-6 single DNA vaccine, the antigen produced substantial protective response while inducing very low antibody and cytokines titers[15]. Immunization with both MPT-32 and PstS-1 constructs evoked strong splenic IFN-γ responses but resulted in no visible protective responses[15]. Although the combination DNA vaccines used by Morris et al.[15] produced higher antibody than that of the current study, our combination seemed to protect the lungs better than theirs. The protective efficacy was 0.82 in their report compared with 2.63 in our work (table 3). The difference in protective efficacy may be a direct result of the combination of DNA vaccines used.

The degree of lymphocyte infiltration may also reflect differences in the immune responses to *M. tuberculosis* infections in various tissue types, as proposed previously[24]. Our study confirmed that the combination of four DNA vaccines improved humoral and cell-mediated immune responses in vaccinated mice and reduced the number of lung CFUs relative to that of controls (plate I). These results indicated that the degree of lymphocyte infiltration may be related to the effectiveness of antigens used as the vaccine. In summary, we have successfully constructed and expressed four bacterial genes encoding secreted proteins from *M. tuberculosis*. Upon co-immunization of these four expression vectors, we found that the new combination DNA vaccines enhanced immune responses and protective efficacy of experimental mice against *M. tuberculosis* infection.

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