

Construction of a DNA vaccine based on the *Mycobacterium tuberculosis* Ag85A/MPT64 fusion gene and evaluation of its immunogenicity

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Abstract. The aim of this study was to construct a DNA vaccine based on the Ag85A/MPT64 gene of *Mycobacterium tuberculosis* (MTB) and analyze its immunogenicity by enzyme-linked immunospot (ELISPOT) assay. The fusion gene encoding Ag85A/MPT64 was amplified by PCR from the genome of the MTB H37Rv strain and cloned into a eukaryotic expression vector followed by confirmation using restriction endonuclease digestion and DNA sequencing. The immunogenicity of the recombinant vector was tested *in vivo* in BALB/c mice. The serum antibody titers against Ag85A/MPT64 were detected by ELISPOT assay. The number of ELISPOT spots for the mice following immunization with Ag85A/MPT64 was significantly greater than for the negative and blank controls. A DNA vaccine based on the gene encoding the Ag85A/MPT64 fusion protein of MTB was successfully constructed and expressed. Our data may serve as a foundation for further research into the prevention and treatment of tuberculosis and carcinomas.

Introduction

In recent years, with the emergence of drug-resistant or multidrug-resistant strains of *Mycobacterium tuberculosis* (MTB), the resurgence of tuberculosis has become a public health and social problem of worldwide concern. According to statistics released by the WHO in 2007, the global annual TB incidence is approximately 9.27 million cases and approximately 130 million people succumbed to the disease. The traditional BCG vaccine is very unstable and the protective immunity it provides to individuals from different parts of the world differ widely (1). Therefore, it is essential that studies

concerning new tuberculosis vaccines and diagnostic reagents are carried out.

A new generation of DNA vaccines has been developed and has shown significant advantages in the prevention and treatment of a variety of infectious diseases and cancers (2). One prerequisite for the development of an effective vaccine is the selection of a highly immunogenic antigen. MTB culture filtrate proteins (CFPs) have been used as antigen compositions for TB prevention and in diagnostic reagents (3,4). It has been found that the Ag85 complex is the major secreted protein antigen of MTB, accounting for 30% of the total secreted proteins (5). The antigen-specific response of CD4⁺ and CD8⁺ T cells to an Ag85A DNA vaccine in BALB/c mice demonstrated that the Ag85A DNA vaccine was able to produce a strong and broad T-cell response and CTL activity (6). MTB secretes MTB protein 64 (MPT64), which is a major secreted protein in the early culture filtrate of MTB, accounting for 8% of total secreted proteins. The MPT64 protein has superoxide dismutase activity and a strong cellular immune activity with a specificity higher than that of tuberculin purified protein derivative (PPD) (7). In 1999, Kamath *et al* reported that the immunization of mice with MPT64 DNA vaccines induced spleen cells to produce IFN- γ and also induced MPT64-specific CTL activity (8).

In order to combine the characteristics of the Ag85 and MPT64 proteins, a DNA vaccine was constructed and expressed in the present study. The recombinant proteins were purified and then validated by enzyme-linked immunospot (ELISPOT) assay.

Materials and methods

Materials. Pfu (*Pyrococcus furiosus*) Taq DNA polymerase, dNTPs, buffer and MgCl₂ were purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The *Eco*RI and *Bam*HI restriction enzymes, T4 DNA ligase kit and DNA markers were purchased from Takara Bio, Inc. (Shiga, Japan). The PCR product purification kit was purchased from Omega Bio-Tek, Inc. (Norcross, GA, USA). The gel extraction and plasmid extraction kits were purchased from Shanghai Huashun Bioengineering Co., Ltd. (Shanghai, China). The bacterial genomic DNA extraction kit was purchased from Shanghai Sangon Biotech Co., Ltd (Shanghai, China). The human IFN- γ

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capture and IFN- γ detection antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Phytohemagglutinin (PHA) was acquired from Sigma (St. Louis, MO, USA).

Animals. A total of 24 6-week-old special pathogen-free female C57BL/6 mice were purchased from the Experimental Animal Center of Jilin University, China. The mice were given free access to food and water throughout the study. The study was approved by the Institutional Animal Care and Use Committee of Jilin University, Changchun, China.

Genomic DNA extraction. Small amounts of MTB H37Rv were inoculated in 7H9 liquid medium and cultured at 37°C for two weeks. The liquid culture (3 ml) was centrifuged at 12,300 rpm for 1 min. The pellet was resuspended in 50 μ l lysis buffer (5 μ l 10X PCR buffer, 5 μ l 45g/l Tween-20, 5 μ l 45 g/l NP-40, 0.5 μ l 20 g/l protease K and 34.5 μ l water) and kept at 55°C for 1 h and then in a boiling water bath for 10 min to inactivate proteinase K. Following centrifugation at 12,300 rpm for 1 min, the supernatant was extracted twice with phenol/chloroform/isoamyl alcohol, followed by extraction twice with phenol/chloroform. The extract was precipitated using pre-cooled ethanol and then frozen at -80°C for 10 min. The precipitate was dissolved in 50 μ l TE and stored at -20°C.

Primers. The Ag85A and MPT64 primers were synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA). According to the gene coding sequences of MTB H37Rv Ag85A and MPT64, four primers were designed: P1, MPT64 forward primer 5'-GGAATTCATGGTGCATCAAGATCTT-3' containing *Eco*RI restriction sites and start codon; P2, MPT64 reverse primer 5'-GGATCCCTAGGCCAGCATCGAGTC-3' containing *Bam*HI sites and stop codon; P3, Ag85A reverse primer 5'-AGGATCCATGTTTTCCCGGCCGGGCTTG-3', containing *Bam*HI restriction sites and start codon; and P4, Ag85A reverse primer 5'-GGAATTCCTATGTTCGGAGCTAGGCGCCCTGGG-3', containing *Eco*RI restriction sites and stop codon. The PCR reaction system consisted of: 5 μ l 10X buffer, 1 μ l dNTPs (2.5 mM), 2 μ l DNA, 0.5 μ l (50 μ M) primer and 0.5 μ l (2 units) Taq DNA polymerase, with water added to 50 μ l. The reaction conditions were as follows: 95°C for 5 min, 95°C for 45 sec, 60°C for 30 sec and 72°C for 60 sec, for 32 cycles, then 72°C for 10 min. The PCR products were identified using 0.8% agarose gel electrophoresis.

Ag85A/MPT64 cloning plasmid construction. Double-digested plasmid pcDNA3.1(+) and the PCR product were ligated by T4 DNA ligase at 22-24°C overnight and then transformed into DH5 α competent cells. The cells were cultured in 2YT medium containing 100 mg/l ampicillin. The blue/white method was used for screening positive clones. White colonies were selected randomly and inoculated into 4 ml liquid 2YT medium containing ampicillin at 37°C with agitation overnight. Plasmid DNA was extracted using the alkaline lysis method. The plasmid DNA was digested with *Bam*HI and *Eco*RI. The Ag85A (978 bp) and MPT64 (738 bp) fragments were detected using 0.8% agarose gel electrophoresis. Positive clones were sequenced by Shanghai Sangon Biotech Co.

Ag85A/MPT64 eukaryotic expression plasmid construction. Positive clones of the Ag85A/MPT64 recombinant plasmid were digested with *Bam*HI and *Eco*RI and the products were analyzed using 0.8% agarose gel electrophoresis. A PCR product of size 1716 bp was recovered using the PCR product purification kit and then dissolved in 35 μ l water and stored at -40°C. At the same time, an eukaryotic expression plasmid was prepared using the same method. Double-digested plasmid and DNA were ligated at 22-24°C overnight and then transformed into BL21 competent cells. The cells were cultured in 2YT medium containing 100 mg/l ampicillin. The blue/white method was used for screening positive clones. Positive clones were identified by *Bam*HI and *Eco*RI double digestion.

DNA vaccine preparation and animal immunization. Following the mass culture of BL21 cells containing the Ag85A/MPT64 plasmid, the plasmid was extracted and then purified using phenol/chloroform/isoamyl alcohol (9). The concentration of plasmid was adjusted to 1.5 g/l with sterile saline. Eight BALB/c mice were injected with 60 μ l 8.5g/l pivacaine in the side of the thigh. Three weeks after pretreatment, the mice were immunized once with 50 μ g Ag85A/MPT64 plasmid. The vector was injected intramuscularly in a 60 μ l volume. The control mice were immunized using the blank plasmid and sterile saline, respectively (8 mice per group).

ELISPOT. Anticoagulant (3-4 ml) was taken from the mouse tail vein, and after centrifugation, the lymphocyte layer was separated from mouse serum, followed by purification of blood mononuclear cells and adjustment of the cell concentration. The positive control (PHA), negative control (normal saline), blank control (plasmid) and different dilutions of the cell suspension were added to a pre-packed IFN- γ capture monoclonal antibody microplate. The cells were cultured at 37°C in 5% CO₂ overnight. The supernatant was discarded and the plates were washed with PBST. The detection antibody was added to each well and the plate was incubated at 37°C for 2 h. After washing, the substrate solution was added to each well and the plate was incubated for a further 30 min. The plates were washed, the reaction was terminated and the results were observed.

Results

Ag85A/MPT64 gene cloning. A specific band was amplified from a template of MTB H37Rv genomic DNA by PCR. Following double digestion, the PCR products were cloned into the plasmid and then transformed into DH5 α competent cells. White colonies were selected randomly and the plasmids were extracted and double-digested. A 978-bp fragment and a 738-bp fragment were obtained (Fig. 1A). Bi-directional sequencing results were consistent with those previously reported (10).

Ag85A/MPT64 vector construction. Plasmid DNA was extracted after positive clones were selected and cultured. Following double enzyme digestion, a 1716-bp fragment was recovered and then ligated with the plasmid fragment that was digested using the same enzyme. Positive clones in the transformed plates were selected randomly. A ~1716-bp fragment

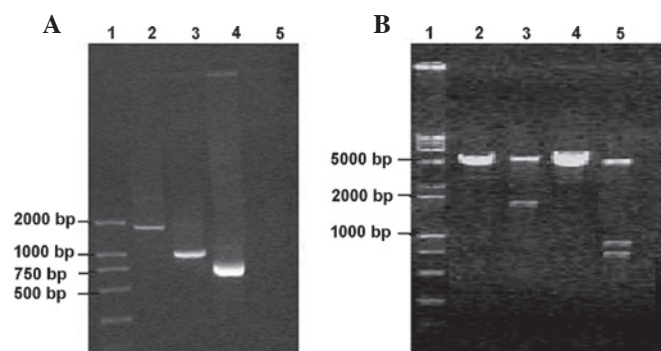


Figure 1. Detection of PCR products and vector restriction sites using agarose gel electrophoresis. (A) Electrophoresis results of the PCR products. Lane 1, DNA marker; 2, PCR fragment of Ag85A and MPT64 fusion gene; 3, PCR product of Ag85A gene; 4, PCR product of MPT64 gene; and 5, negative control. (B) Electrophoresis results for vector digestion products. Lane 1, DNA marker; 2, product of plasmid pcDNA3.1 (+) by single *EcoRI* digestion; 3, product of positive clone by single *EcoRI* digestion; 4, product of plasmid pcDNA3.1 (+) by double digestion; and 5, product of positive clone by double digestion.

was obtained after the plasmid was digested with *EcoRI* and ~978-bp and 738-bp fragments were obtained after the plasmid was double-digested (Fig. 1B). The nucleotide sequence of the recombinant gene encoding Ag85A/MPT64 had no mutations, and the gene was correctly inserted into the vector.

ELISPOT. As shown in Fig. 2, the numbers of ELISPOT spots for the mice immunized with Ag85A/MPT64 were significantly greater than for the negative and blank controls. The vaccine-stimulated production of immunoglobulin (Ig) and cytokines may be screened effectively by ELISPOT assay (11), which is important for vaccine research. The soluble protein in the cells forms clearly visible spots through a color reaction and these spots may be directly and manually counted under a microscope. The spots may also be counted by an ELISPOT analysis system and screened by high-throughput screening, which is far more efficient than other detection methods.

Discussion

A 4,400-kb fragment of MTB contains more than 4,000 genes and these genes encode proteins that may cause strong cellular and humoral immune responses. Therefore, these genes, including HSP, MPT64, Ag85, Esat-6 and PstS-3, may be considered as candidate genes for DNA vaccines (12).

The Ag85 complex includes three subunits, Ag85A, Ag85B and Ag85C, with relative molecular weights of 30×10^3 – 32×10^3 kDa, which exist in the MTB and BCG cell walls and culture filtrates. The Ag85 content is highest in the MTB cell wall proteins and secreted proteins and comprises 31–45% of the total protein (13). Ag85A and PstS-3 DNA vaccines have been used to treat C57BL/6J mouse tuberculosis infection and it was found that DNA vaccines can prevent the recurrence of latent tuberculosis infection in mice and shorten the cycle of conventional chemotherapy. Significant reductions in the load of MTB in the lung and spleen occurred when a Ag85A DNA vaccine was used alone or in combination with chemotherapy to treat multidrug-resistant tuberculosis in

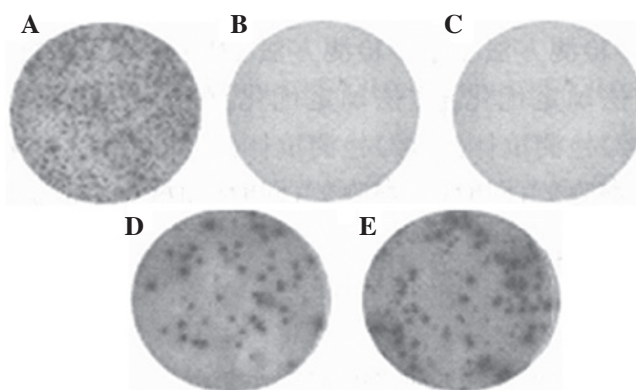


Figure 2. ELISPOT detection of the color reaction of DNA vaccine. (A) positive control; (B) saline negative control; (C) plasmid blank control; (D and E) experimental group with different dilutions of the serum. ELISPOT, enzyme-linked immunospot.

mice for 2 months (14). MPT64 is a major secretory protein of the MTB complex group and is also a key T-cell antigen. MPT64 and ESAT6 DNA vaccines were used to immunize mice and, by observing the pathological changes in the lungs of the MTB-infected mice 4 weeks after abdominal challenge to evaluate the protective strength of the DNA vaccine, it was found that the changes in the MPT64 and ESAT6 DNA vaccine groups were similar to those in the BCG group. This is because most of the mice experienced the proliferative response and a small number of the mice experienced a tissue reaction.

Ag85 is abundant in the cell wall and culture filtrates of MTB and BCG cells. The mycolic acid transferase activity of Ag85 has an important role in the later stages of the synthesis of the MTB cell wall. Ag85A, Ag85B and Ag85C have highly homologous sequences (13). The Ag85/MPT64 protein is capable of stimulating the body to produce a large amount of IFN- γ and induces strong T-cell proliferation. Mice were able to resist MTB attack following immunization with Ag85/MPT64 (15). It has also been reported that high levels of Ag85/MPT64 antibodies were detected in the serum following the infection of individuals with TB bacilli (16).

Antigens are a feasible alternative to BCG. When BCG enters the bladder, it combines with the tumor cell surface fibronectin (FN) or bladder epithelial cells and causes an immune response. This combination confers the characteristics of the Ag85 complex, known as the fibronectin-binding antigen (12,13). The Ag85 protein was expressed in some regions of the bladder following the infusion of a eukaryotic expression vector into the bladder. Therefore, it appears that the bladder wall absorbs the eukaryotic expression vector DNA vaccine.

An Ag85A/MPT64 DNA vaccine has been successfully constructed in this study and it was confirmed that specific antibodies against MTB were produced following animal immune challenge.

ELISPOT assays are more specific and sensitive than TST, which is helpful for the screening and monitoring tuberculosis infection of patients undergoing anti-tumor necrosis factor (TNF) treatment (17). A IFN- γ test using TB-specific antigens enables the efficient diagnosis of latent TB infection (LTBI) in HIV-infected individuals. ELISPOT assays with their

high sensitivity and specificity play an important role in TB screening. Due to the high incidence of tuberculosis and BCG vaccination in China, investigations may be carried out using this technology among tuberculosis-exposed populations to exclude the effect of TST and PPD false positives and false negatives and for the early diagnosis of tuberculosis-infected individuals. According to the degree of reaction, preventive treatment may be carried out if necessary, which will reduce the incidence and the source of infection.

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