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Complex determination of immunogenicity of mycobacterium-proactive and genetic overview and analysis

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ABSTRACT

Tuberculosis is a serious infectious disease caused by Mycobacterium tuberculosis. DNA vaccination is an advanced technique for protecting human bodies from infectious diseases including tuberculosis by injecting exogenous gene engineering DNA into the body to produce an immunological response. This research mainly focused on the immunogenicity of new DNA vaccines: pVAX1-64. Recombinant vector pVAX1-64 was constructed, identified successfully and then transfected into BHK-21 cells and screened of stable expressing cell lines. Specific antibody titer test in serum of immunization mice and tests for allergic reaction in immunization guinea pigs were performed. Finally $CD4^+$ ▪ $ACD8^+$ T cells in immunization mice were detected. The antibody titer of MPB in the immunized mice serum was obviously increased significantly with the increased immune days($p<0.01$). Compared with saline group and pVAX1 group, there were significant increased on the number of $CD4^+$ and $CD8^+$ T cells in BCG and pVAX1-64 group ($p<0.05$). There was no allergic reaction after the immunization in guinea pigs, which were sensitive to mycobacteria, using the recombinant expression plasmid pVAX1-64. This new DNA vaccines would not interference the PPD test. All these suggested that this new DNA vaccines is an ideal vaccine and may be further developed as a useful method to prevent tuberculosis.

KEYWORDS

Tuberculosis;
DNA vaccines;
pVAX1-64;
Recombination plasmid;
Antibody titer;
$CD4^+$ T cells;
$CD8^+$ T cells;
Allergic reaction.

INTRODUCTION

Tuberculosis is a common, serious and sometimes lethal infectious disease caused by Mycobacterium tuberculosis (M. tuberculosis). Tuberculosis usually attacks the lungs, while sometimes it also attacks other organs of human body such as bone, ovary and stomach[11]. The main distribution of tuberculosis is located in many Asian and African countries. The most common vaccine against tuberculosis currently is Bacillus Calmette-Guérin (BCG), prepared from a strain of the attenuated or weakened live bovine tuberculosis bacillus. However, the protection effect of BCG sometimes is not certain, sometimes the immune results are not well as expected[18]. What is more, BCG could interfere the test of Purified Protein Derivative (PPD,

also known as the Mantoux screening test), a diagnostic tool for tuberculosis, making the PPD test confused[10]. Moreover, it makes the routine quarantine more difficult after vaccinated with BCG since natural infection and artificial immunity would be undistinguished. And the safety of BCG is still need to be improved. So it is very important to discover the new vaccine for the diagnosis and prevention of the tuberculosis. DNA vaccines are one of the ideal vaccines. They are third generation vaccines, made up of a plasmid containing exogenous genetically engineered DNA in order to produce antigens[9, 14]. DNA vaccines elicit an ideal immunological response when highly active expression vectors used and thus result in an ideal protection for human bodies[8]. No risk for infection, long-term persistence of immunogen and focused immune response are the advantages of new DNA vaccines compared with the other traditional vaccines[5, 12, 16]. What's more, ease of development, production, storage and shipping, together with cost-effectiveness, are also the advantages of new DNA vaccines[6]. Thus, it is important to design a new DNA vaccines to better prevent tuberculosis. This is the main task and purpose of this research.

MATERIALS AND METHODS

Construction of recombinant vector pVAX1-64

The recombinant vector pGEM-T-64 was amplified in *E.coli* JM109 and pVAX1 in E.coli DH5. Vectors of pGEM-T-64 and pVAX1 were double digested with BamH I and EcoR I. Target gene MPB64 was purified and then connected with expressing vector pVAX1 with T4 Ligase. The recombinant vector pVAX1-64 was amplified in *E. coli* DH5α and then extracted by a DNA purification kit (Promega) according to the instructions. The designed vector was retrieved from thegel. pVAX1-64 was double digested by BamH I and EcoR I and then evaluated by 0.8% agarose gel electrophoresis. PCR was used to detect MPB64 using the recombinant vector pVAX1-64 as template. The purity of the recombinant vector was detected by ultraviolet spectrophotometer and evaluated according to OD260/OD280.

Transfection of BHK-21 cells and screening of stable expressing cell lines

BHK-21(ATCC) cells were transfected with pVAX1-64 and pVAX1by Lipofectamine 2000 (Invitrogen, USA). Cells at 90–95% confluence were transfected with 5μg vectors. After 6-hours exposure, the normal culture medium,RPMI 1640 supplanted with 15% horse serum (Gibco, USA) was added into cells for another 18h-42h. Tuberculosis polyclonal antibody (1:50) was used as 1st antibody while FITC marked Rabbit anti bovine IgG(1 ▪ F16)was used as 2nd antibody, each for 2h at 37°C. Pictures were taken under fluorescence microscope. Transfected cells were collected and total RNA was isolated and then translated into cDNA as template. RT- PCR was performed for gene detection.

Detection of antibody titer in serum in immunized mice

Blood were collected 3 weeks after immunization from mice tail. Serum were separated and stored at -20°C. ELISA was used to detected the antibody titer in serum. MPB was diluted to a final concentration of 10 μg/ml in Coating Buffer. 100 μl solution were transfered to each well of a high affinity, protein-binding ELISA plate and incubated at 4°C overnight. Solution was flicked off and washed 3 min/ time X 3 times with TPBS, and blocked using 100 μl of 1% BSA in each well and incubated at 37°C for 1 hour. Solution was flicked off and washed 3 min/ time X 3 times with TPBS, Serum were diluted to desired concentrations (1 : 40, 1 : 80, 1 : 120, 1 : 160, 1 : 200…) in Blocking Solution and 100 μl were added per well to the ELISA plate and incubated at room temperature for 2 hours. Solution was flicked off and washed 3 min/ time X 3 times with TPBS. The HRP-labeled rabbit anti mouse Ig G was diluted (1:500) in Blocking Solution. 100 μl of diluted antibody were added to each well and incubated at 37°C for 2 hours. Solution was flicked off and washed 3 min/ time X 3 times with PBS/Tween. 100 μl of substrate solution was added and incubated at room temperature for 30 min avoiding light. 50 μl of suspending solution were added and the optical density (OD) for each well was recorded with a microplate reader set to 492 nm.

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Detection of $CD4^+$ ▪ $ACD8^+$ T cells in immunizated mice

The suspension of lymphocytes of spleen was centrifuged at the speed of 4000r/min for 10 min. The supernate was flicked off and washed with PBS(pH 7.2) twice and suspended in PBS. Flow cytometry(FCM) was used to detect the $CD4^+$ ▪ $ACD8^+$ T cells.

Allergic reaction in guinea pigs

Intracutaneous injection of PPD was performed on guinea pigs 21 days after last immunization. The allergic reaction was tested 24h, 48h, 72h after injection. The total volume was 15ul and bulb appeared after injection. The tumefacient area was measured and calculated using vernier caliper. Another allergic reaction was performed 1 month after the first one.

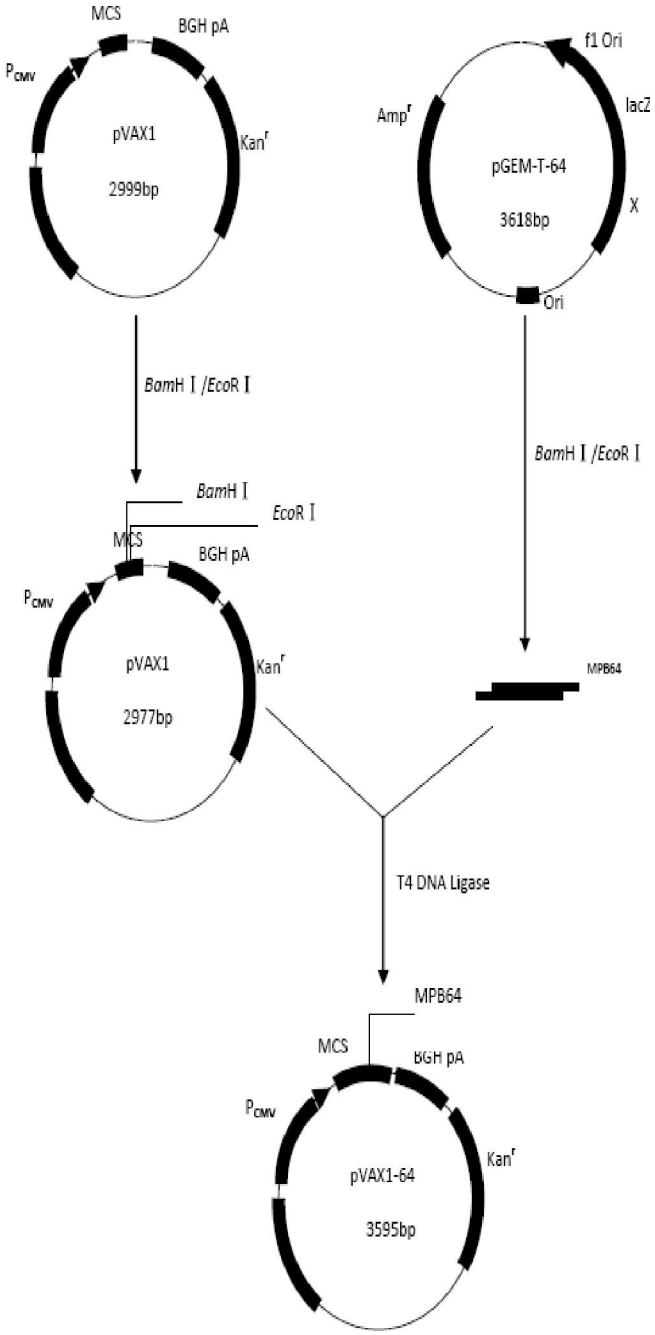
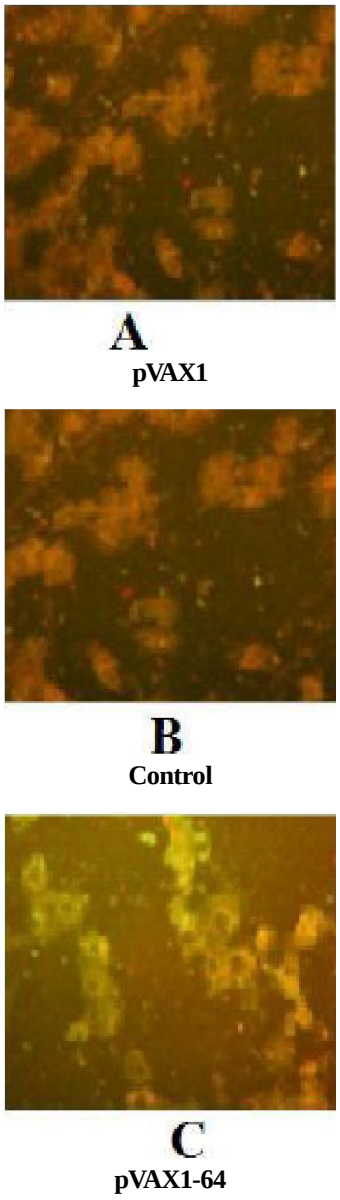


Figure 1 : The recombination expressing plasmid Pvax1-64

RESULTS

We first give the Figures and TABLES tha potray our results and the discussions that follow.



A
pVAX1

B
Control

C
pVAX1-64

Figure 2 : Fluorescence antibody assay for BHK-21 cell transfected with recombinantion expression plasmids

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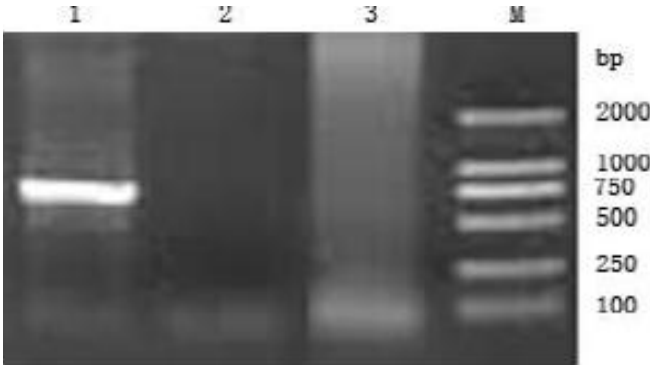

Figure 3 : RT-PCR product of BHK-21 transfected with pVAX1-64

Lan1 ▪ FRT-PCR product of BHK-21 transfected cell of pVAX1-64; Lan2 ▪ FRT-PCR product of normal BHK-21 cell; Lan3: RT-PCR product of BHK-21 cell transfected with pVAX1 M ▪ FDNA Marker/DL2000

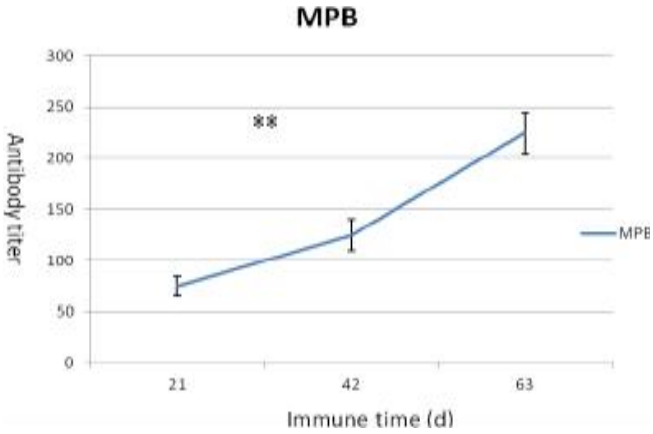

Figure 4 : Antibody titer in serum of vaccinated mice (pià0.01)

TABLE 1 : Detection of CD_4^+ and CD_8^+ T cells of the immunized mice

Group	Samples	$CD4^+$ T cell (%)	$CD8^+$ T cell (%)
pVAX1-64	10	26.99 ± 0.310^a	19.36 ± 0.44^a
BCG	10	27.58 ± 0.410^a	21.42 ± 0.51^a
pVAX1	10	17.11 ± 0.22^b	9.180 ± 2.67^b
Saline	10	15.25 ± 0.41^b	8.770 ± 2.37^b

a▪ Cb▪ Cc▪ Cd Data with significant differences in the same line ▪ Cpià0.05

TABLE 2 : Allergic reaction in guinea pigs

Time	Group	Samples	Tume facient areaΦmm) 24h	Tume facient areaΦmm) 48h	Tume facient areaΦmm) 72h
1	BCG	6	6.3406 ± 0.7399^A	9.1752 ± 0.7945^A	10.1283 ± 0.6972^A
	pVAX1-64	6	2.5249 ± 0.8362^B	1.5413 ± 0.3831^B	0.6123 ± 0.4462^B
	pVAX1	6	2.6838 ± 0.2690^B	1.5724 ± 0.3284^B	0.6769 ± 0.3125^B

A,B Data with obvious significant differences in the same line,,p©, 0.01

Result of detection for BHK-21 Cells transfected with vectors

As shown in Fig.2, the transfected cells were detected by fluorescence antibody test. Specific kelly fluorescence could be detected in BHK-21 translated with recombination expressing plasmid pVAX1-64, while no specific kelly fluorescence could be detected in BHK-21 translated with pVAX1 control vector. This means the MPB64 was expressed in BHK-21. RT-PCR was used to detect the transfection. As shown in Fig.3, the DNA band, with the same base pairs of target DNA, could be detected in BHK-21 translated with recombination expressing plasmid pVAX1-64, while no band could be detected in BHK-21 translated with pVAX1 control vector and normal BHK-21 cells.

Result of antibody titer detection in mice serum

The MPB expressed and purified by the recombination expressing plasmid pVAX1-64, was used as antigen. ELISA was used to detected the antibody titer in the mice serum. The result of titer was shown in Fig.4. The antibody titer in the serum was obviously increased significantly with the increased immune days ($p<0.01$).

Result of numbers of $CD4^+$ ▪ A$CD8^+$ T cells in immunization mice

The lymphocytes of spleen in mice were separated sterilely 3 weeks after the 3rd immunization and detected for the numbers of $CD4^+$ ▪ A$CD8^+$ T cells. As shown in Tab.1, compared with saline group and pVAX1 group, there were significant increased on the number of $CD4^+$ and $CD8^+$ T cells in BCG and pVAX1-64 group. ($p<0.05$).

Result of allergic reaction in guinea pigs

As shown in Tab.2, compared with the BCG group, there were obviously significant differences on allergic reaction in pVAX1-64 and pVAX1 group ($p<0.01$), while no obvious differences between these two groups. Within 24-72h after intracutaneous injection of PPD, the tumefacient area was decreased in pVAX1 group and pVAX1-64 group, while it is opposite in BCG group. All these indicated that there was no allergic reaction after the immunization in guinea pigs, which were sensitive to mycobacteria, using the recombinant ex-

pressing plasmid.

DISCUSSION

Tuberculosis(TB) is a common and sometimes lethal infectious disease caused by various strains of Mycobacterium tuberculosis(MTB). The main cause of TB, MTB, is a small aerobic non-motile bacillus(G^+). MTB can withstand weak disinfectants and survive in a dry state for weeks[20]. Tuberculosis usually attacks the lungs but can also affect other organs of the body such as bone and stomach. It is usually spread through the air. Most infections in humans is asymptomatic and latent, if untreated, some infected person would be dead. Strains of tuberculosis with antibiotic resistance, sometimes with multi-drug-resistant is growing. Prevention relies on screening programs and vaccination, such as Bacillus Calmette-Guérin vaccine(BCG)[1].

Vaccines are one of the greatest achievements of modern medicine. It is one of the most effective methods to prevent zymosis including tuberculosis. DNA vaccination is a new technique for protecting the human body from epidemic diseases by injecting genetically engineered DNA, also called foreign DNA, into bodies to produce an immunological response, the best immune response when highly active expression vectors are used[15]. These expression plasmids which usually consist of a strong promoter to drive the in vivo transcription and translation of the target gene, optimising vector design for maximal protein expression is essential and important. They could elicit the body to produce certain antibody to protect itself. These new vaccines have a number of advantages over conventional vaccines, including the ability to induce a wider range of immune response types, no risk for infection, long-term persistence of immunogen and focused immune response[4]. An antibody titer is a common measurement of quantity of antibody in human body has produced that recognizes a particular epitope, expressed as the greatest dilution that still gives a positive result[3]. ELISA is a common used method of determining antibody titers. Our research found that the MPB expressed and purified by the recombination expressing plasmid pVAX1-64 could be enough to produce an effective immunological response. Immunogens could be targeted to various cellular compartments in order to improve antibodies or cytotoxic T-cell responses. In response to an antigen, cell-mediated immunity involves the activation of natural killer cells (NK), macrophages, antigen-specific cytotoxic T-lymphocytes, and cytokines, instead of antibodies. The immune system was separated into humoral immunity and cellular immunity, for which the protective function of immunization was associated with cells[7, 13]. Cell-mediated immunity is directed primarily at microbes survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in defending against fungi, protozoans, intracellular bacteria, and cancers. It also plays a major role in transplant rejection in human body[2]. $CD4^+$ T cells ▪ Calso known as T helper cells (Th cells), a subgroup of lymphocytes, plays an important role in establishing and maximizing the capabilities of the immune system. These cells provide signals for orchestrating the immune response. Th cells are involved in activating and directing other immune cells and are particularly important in the immune system[11, 22]. $CD8^+$ T-cells, a cytotoxic T cell, also known as killer T cell, belongs to a sub-group of T lymphocytes. $CD8^+$ T-cells, recognized as TC cells once they become activated and are generally classified as having a pre-defined cytotoxic role within the immune system, can kill tumor cells, cells infected with viruses or other pathogens, or damaged or dysfunctional[22]. $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells recognize antigen in a complex with either type 1 or 2 major histocompatibility complex. Our research found significant increased on the number of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells in BCG and pVAX1-64 group, indicating the immunological response occurred via cellular immunity or called cell-mediated immunity[17]. Allergic reactions, a hypersensitivity disorder of the immune system, occur when a person's immune system reacts to an allergen. Allergic reactions are distinctive because of excessive activation of mast cells and basophils by IgE[21]. The results of allergic reaction in guinea pigs induced by PPD indicated that the differences among pVAX1-64 group, pVAX1 control group and BCG group were significant obviously (Pià0.01), this meant that immunization in pVAX1-64 group would not induce allergic reaction, as well as pVAX1 control group. This results were coincident with the reports from Vordermeier et al and

Baldwin et al. This result means pVAX1-64 eukaryon recombinant expression vector is an ideal vaccine since it would not induce allergic reaction and would not interference the PPD test. Our lab has generated eukaryon recombinant expression vector pVAX1-64 successfully by inserting the MPB64 from pGEM-T-64 into expressing vector pVAX1. These results indicated that pVAX1-64 eukaryon recombinant expression vector could stimulate the body to produce special antibody induced by MPB64 and $CD4^+$,$CD8^+$ T-cells-mediated immunity. This vector would not induce allergic reaction. This new DNA vaccine is safe and may better prevent the tuberculosis.

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