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Full Length Research Paper

Immunogenicity of a tuberculosis DNA vaccine expressing a fusion protein Ag85B - MPB64 in mice

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Tuberculosis remains a major infectious disease worldwide. There are no effective vaccines developed for the disease so far and the efficacy of the only available vaccine, *Mycobacterium bovis Bacillus* Calmette–Guérin (BCG) is generally low against *Mycobacterium tuberculois* (MTb). DNA vaccine is especially becoming promising and novel vaccine approach. In this study, a plasmid DNA vaccine, pVAX1-MPB64-Ag85B (pMB), was constructed and designed to express a fusion protein MPB64, Ag85B of MTb. Its immunogenicity and protective efficacy were assessed in a mouse model of tuberculosis. Vaccination with the pMB significantly increased the frequency of peripheral blood $CD4^+$ and $CD8^+$ T cells, and induced significantly higher levels of cell-mediated immune responses, as compared with vaccination with PBS or the pVAX1 empty vector. High levels of antibodies observed in the sera of immunized mice depicted strong humoral responses generated by DNA vaccine constructs. All the experimental vaccines imparted significant protection against challenge with *M. tuberculosis* H37Rv as compared to vector controls. These results show that the newly developed pMB vaccine may be used for the prevention and therapeutic intervention of MTb infection.

Key words: *Mycobacterium tuberculosis*, Ag85B , MPB64, DNA vaccine.

INTRODUCTION

Tuberculosis (TB) remains a leading infectious morbidity and mortality worldwide. Currently, approximately one-third of the total population in the world is infected with *Mycobacterium tuberculosis* (M.tb) and 5–10% of people will develop active TB during their lifetime. It is estimated that there are nine million new cases of active TB with two million deaths annually (Sala and Hartkoorn, 2011; Hanta et al., 2012). Currently, the *Mycobacterium* bovis Bacillus Calmette–Guérin (BCG) is a live attenuated strain of *M. bovis* and is the only available TB vaccine obtained almost one century ago. Although vaccination with BCG has shown to prevent the severe MTb-related meningitis and miliary TB in children, it has poor protection against adult pulmonary TB with variable efficacies ranging from 0 to 80% (You et al., 2012). Consequently, there is now a concerted effort to look for other promising and effective vaccine candidates to combat TB. Currently, DNA vaccines are under intensive investigation, because both long-lived humoral and cellular immune responses can be engendered by a DNA vaccine (Kuo et al., 2012).

Among secretory proteins of *M. tuberculosis*, members of the Ag85 complex (Ag85A, B, and C) have been evaluated most extensively as vaccine candidates (Kaufmann et al., 2010). A level of protection superior to that given by BCG has been demonstrated to be induced by vaccination with recombinant BCG overexpressing Ag85B (You et al., 2012). These findings argue strongly in favor of Ag85B as an important candidate for a tuberculosis subunit vaccine. BCG vaccination of humans, however, induces T cell responses to the Ag85 complex, but protection remains incomplete. It is therefore envisaged that in order to achieve more effective induction of cell-mediated immunity, including memory immunity, additional antigens may be required. Some of these important T cell antigens could be located within the missing genomic portions of BCG (You et al., 2012; Hawkridge and Mahomed, 2011). Numerous

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studies have demonstrated that the antituberculosis vaccine potential of ESAT-6 proteins are absent in BCG (Delogu and Fadda, 2009).

According to available information, co-immunization using the plasmids encoding protective antigens induces a greater degree of protection over a vaccine consisting of a single immunodominant antigen (Sala and Hartkoorn, 2011). In this regard, a combination of Ag85 protein and immunodominant proteins encoded by regions of difference(RD) antigens can represent a broad epitopic repertoire that leads to activation of helper T cell and cytotoxic T cell responses. So far, various multivalent combinations of DNA vaccines encoding Ag85 complex proteins along with several immunodominant secretory proteins have been evaluated (Rouanet and Locht, 2010; Kalra et al., 2007). However, no reports are available on the use of a multivalent combination of DNA vaccines based on RD encoded MPB64 along with the Ag85 complex proteins against tuberculosis. Thus in this study, we evaluated the DNA- based Ag85B- MPB64 vaccine candidates for their immunoprophylactic potential against M.Tb.

MATERIALS AND METHODS

Animals

Pathogen-free C57BL/6 female mice were obtained from the Laboratories center of Jilin University (Changchun, China). The mice were maintained under barrier conditions and fed commercial mouse chow and water *ad libitum*. The mice were 6 to 8 weeks old at the time of the vaccinations.

Construction of the pMP64/85 DNA vaccine

The entire Ag85B gene sequence from M.tb H37Rv chromosome DNA was amplified by PCR and subcloned into the eukaryotic expression vector pVAX1 (Invitrogen,USA). The primer sequences were (forward) 5′-TGAATTCAAATGTTCTCCCGGCCG-3′ and (reverse) 5′-AAAGGATCCCAGC CGGCGCCTAA-3′, where the underlined oligonucleotides represent *EcoRI* and *BamHI* sites, respectively, to facilitate cloning. The amplified products were purified with the Tiangen gel extraction kit (Tiangen, China), digested with *EcoRI* and *HindIII* and ligated into the eukaryotic expression vector pVAX1 that was digested by *EcoRI* and *BamHI* to yield the recombinant plasmid pVAX1-Ag85B. The entire MPB64 gene sequence of M.tb H37Rv was also successfully amplified by PCR. MPB64 was digested with *HindIII* and *BamHI* and ligated to the eukaryotic expression plasmid pVAX1- MPB64 -Ag85B, which was also digested with *HindIII* and *BamHI* to yield the eukaryotic expression plasmid pVAX1-ESAT6-Ag85B (called pMB). The plasmids pVAX1-Ag85B (also called p85B) and pMB were confirmed by DNA sequence analysis.

Indirect immunofluorescence analysis

Six-well tissue culture plates were seeded with COS-7 cells (10^6/well). Monolayer of 80 to 90% confluent cells was transiently transfected with the plasmid pMB and empty plasmid by using Lipofactamin Reagent (Invitrogen,USA). 36 h after transfection, cells were washed with phosphate-buffered-saline (PBS), fixed with100% acetone for 10 min at -20 °C and washed once again with PBS. Diluted primary and secondary antibodies were incubated at 37°C for 1 h, respectively. Primary antibodies used were antiserum of rabbit to Ag85B(Sigma, USA), and secondary antibodies were FITC-conjugated–goat- anti-rabbit IgG (Sigma, USA).

Immunization of mice with DNA vaccine

The plasmids p85B, pMB and pVAX1 amplified in *Escherichia coli* DH5a were extracted using the alkaline lysis method, then purified by PEG8000 precipitation. For DNA immunizations, mice were randomly divided into five groups (n = 20 each).The mice were immunized intramuscularly with 100 µg of plasmid pMB (group 1), p85B(group2), and 100 µg of empty plasmid pVAX1 (group 3). The BCG control group was vaccinated with 5×10^6 CFU of BCG subcutaneously (group 4), and mice injected with 0.2 ml PBS as control (group 5). All groups were boosted with an equivalent dose at 14 days after the initial inoculation.

Measurement of antibody levels by ELISA

Sera were collected after booster vaccination until challenge, and pre-vaccination sera were also collected. Total serum immunoglobulinG (IgG) specific for M.tb was measured by indirect enzyme-linked immunosorbent assay (ELISA). The test sera were diluted by 1:100 and then manipulated according to the instruction of M.tb antibody detection ELISA kit (Kanghua, China), and the optical density at 450nm were measured in an ELISA microplate reader. Every test serum was run in triplicate in each assay, as well as including negative and positive control sera.

Analysis of $CD4^+$ and $CD8^+$ T-lymphocytes

Peripheral blood samples from immunized mice were collected from 7 days after the booster. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque density gradient centrifugation and adjusted to 1×10^7 cells/ml. 100 µl of cell suspensions (1×10^6 cells) was incubated for 1 h at room temperature with both mouse anti-mice CD4-PE and mouse anti-mice CD8-FITC (BD Biosciences Pharmingen) simultaneously. The samples were processed on fluorescence activated cell sorter.

Experimental infections

Two weeks after the final immunization, mice were infected via the lateral tail vein or intransally (i.n.) with an inoculum of 5×10^6 CFU of M. tb H37Rv suspended in 0.1 ml PBS according to Anderson et al. (2001). All of the mice were examined daily for 2 weeks for the clinical symptoms such as coughing,sneezing, ataxia, dyspnea or death.

Statistical analysis

Statistical analysis was performed using Mann–Whitney's U test and the Kruskal–Wallis test. Values were expressed as the mean ± SD ($p < 0.05$). In general, p values less than 0.05 were considered statistically significant.

RESULTS

Construction of recombinant vaccine expressing MPB64-Ag85B fusion gene

To construct the recombinant plasmids, the DNA

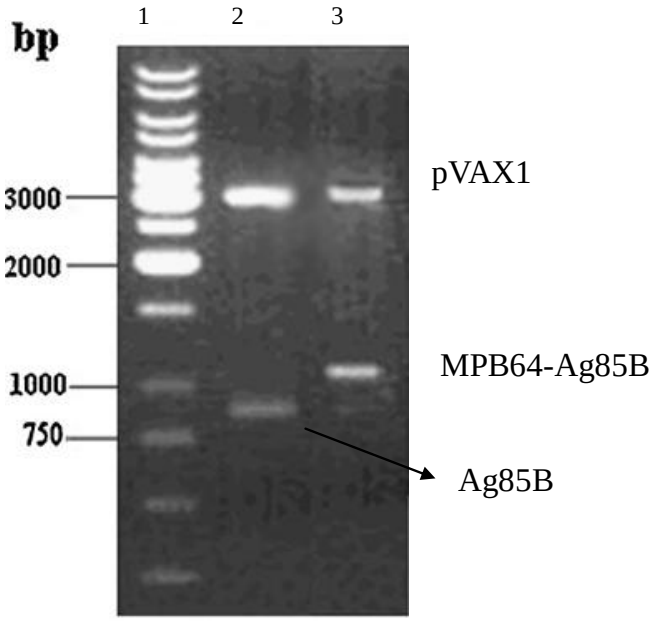

Figure1. Analysis of plasmid constructs. Restriction enzyme digestion analysis of recombinant plasmids pVAX1-Ag85B (*BamHI* and *EcoRI* digestion, Lane2), and pVAX1-MPB64-Ag85B (*HindIII* and *BamHI* digestion, Lane3); Lanes1, DNA marker.

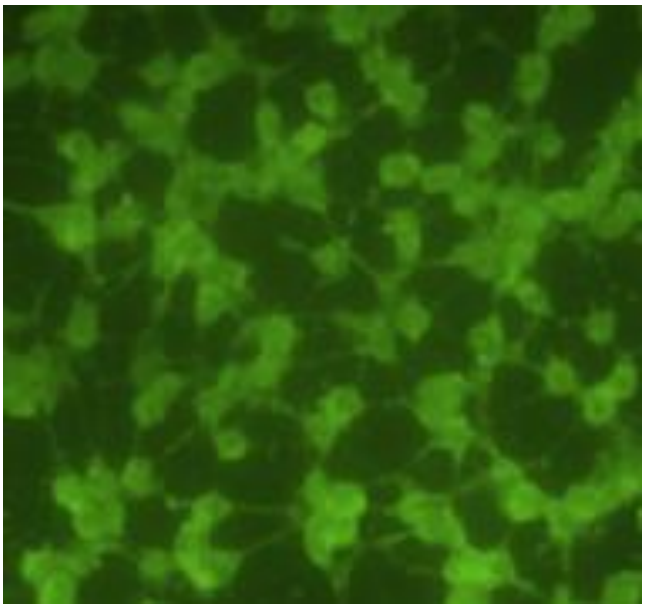

Figure 2. Indirect immunofluorescence detection of the expressed chimeric protein in COS-7 cell. Cells transfected with plasmid pMB showed positive results.

fragment of the entire Ag85B gene sequence from M. tb H37Rv was amplified and cloned into the eukaryotic expression vector pVAX-1. The DNA fragment of the entire MPB64 gene sequence was amplified from M. tb H37Rv and then inserted into pVAX1-Ag85B to yield the recombinant plasmid pMB . Restriction enzyme digestion analysis (Figure 1) showed that the relative molecular mass (Mr) of each inserted DNA fragment was identical to the value predicted.

Expression of recombinant plasmids in COS-7 cells

The expression of pMB was demonstrated by indirect immunofluorescence assay. The cells transfected by pMB displayed fluorescence in cytoplasm (Figure 2), which showed constructs encoding MPB64 and Ag85B fusion protein was expressed successfully in the eukaryotic system.

Humoral immune reaction induced by DNA vaccine

To determine the antibody responses against the recombinant fusion DNA vaccine, BALB/c mice were randomly divided into four groups and immunized with the recombinant vaccine. Two weeks after the immunization, levels of Ag85B-specific IgG in the sera of immunized mice were determined via ELISA. It was found that the plasmid p85B, pMB and BCG significantly increased antibodies in mice 2, 4 and 6 week after booster, respectively, while there were no detectable specific antibodies in the plasmid pVAX1 group and PBS group (Figure 3). The antibody titers were similar in the pMB group and the BCG group after booster. Additionally, the antibody titers of BCG and pMB group were higher than p85B, which showed that the two groups can induce the strongest IgG antibody responses among all groups.

Cellular immune reaction induced by DNA vaccine

Peripheral blood lymphocytes were analyzed by flow cytometry two weeks after the booster. The percentage of $CD4^+$+$CD3^+$ and the percentage of $CD8^+$+$CD3^+$ T-lymphocytes in pMB vaccinated group were significantly higher ($P< 0.05$) than those of the pVAX1 group and PBS group, respectively, and also higher than that of BCG group (Table1). The percentage of the two T-lymphocyte subgroups had no significant difference between pVAX1 and PBS group, respectively.

DNA vaccine induced protective immunity against M. Tb H37Rv infection

To evaluate protective responses after M. tb challenge, immunized mice were infected with M. tb H37Rv. The recombinant pMB vaccine groups, and the BCG group had longer 50% death times (T_{50}) and lower death mice compared to the DNA vaccine Ag85B group, the pVAX1 group and the non-immunized PBS group (Table 2). This indicated that the pMB vaccines can induce stronger

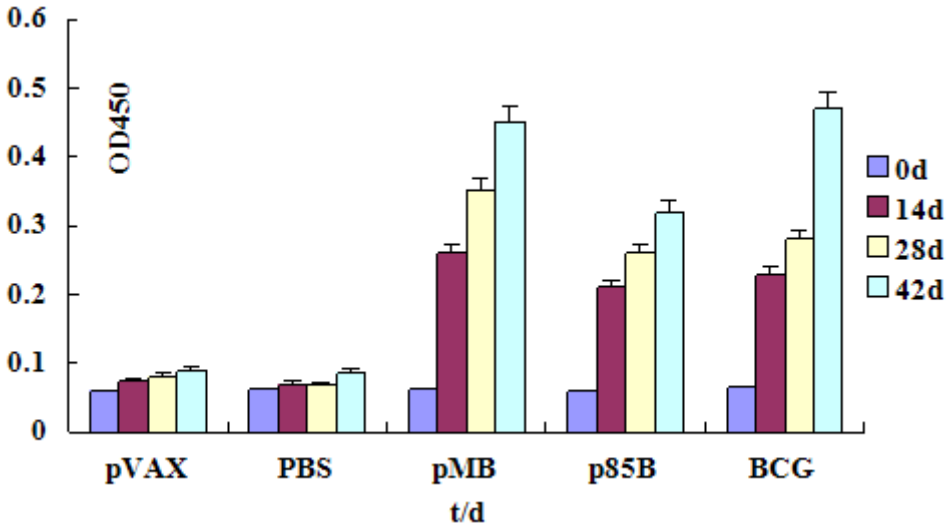

Figure 3. Humoral responses in immunized mice. Peripheral blood anti-Ag85B ELISA antibody levels of immunized mice. Sera from all of mice were sampled 2,4 and 6 weekly post booster. The result was obtained from average of five sera in each group every assay. The data of antibody titers was analyzed by software of Statistics Package for Social Science (SPSS).

Table 1. The percentage of $CD4^+$+$CD3^+$ and $CD8^+$+$CD3^+$ T-lymphocytes of different inoculated groups.

Group	Sample	$CD4^+ + CD3^+$ (%)	$CD8^+ + CD3^+$ (%)
pMB	20	26.48±0.318[a]	16.46±0.45[a]
BCG	20	25.54±0.402[a]	15.49±0.41[a]
p85B	20	20.55±0.314[ab]	12.43±0.39[ab]
pVAX1	20	14.66±0.221[b]	9.180±0.47[b]
PBS	20	13.27±0.208[b]	8.47±0.33[b]

This test was performed 14 days after boosting immunization; different letter represent the significant difference at $p<0.05$.

Table 2. Comparisons of various vaccines-induced protection efficacy in mice infected with H37Rv.

Group	T_{50} (days)	Mortality (%) (50 days)	Sample
pMB	>60	15	20
BCG	>60	20	20
p85B	38	33.33	20
pVAX1	32	80	20
PBS	27	75	20

T_{50}, time when 50% mortality occurred.

protective immunity against M. tb than the single gene vaccine.

DISCUSSION

Currently, great progresses have been made in the development of novel TB vaccines, such as attenuated live or inactive whole bacterium, recombinant BCG, subunit vaccine, and DNA vaccine (Hoft, 2008). DNA vaccines can induce substantial cellular immunity against TB and can invoke both CD4 and CD8 T cell responses compared with conventional vaccines (Oslen and Andersen, 2003; Britton et al., 2003). Compelling

evidence exists from both humans and murine studies that protection against TB mainly depends on antigen-specific Th1 responses (Quesniaux et al., 2010). Indeed, DNA vaccine preferentially induces Th1dominant immune response. However, the efficacy of DNA vaccines for expressing single M.tb antigen in protection against TB is limited (Fan et al., 2009). Hence, new strategies are demanded to improve the protective efficacy of DNA vaccines against TB. In the present study, we generated a new plasmid, pMB, for expressing a fusion protein of Ag85B and MPB64, and found that vaccination with the plasmid pMB induced potent antigen-specific Th1 responses.

$CD4^+$ and $CD8^+$ T cells are crucial for defending intracellular pathogens. In addition, $CD8^+$ T cells can also produce perforin and granulysin, which can directly kill the M.tb-infected cells and attack M.Tb (Woodworth et al., 2008).It was found that the frequency of peripheral blood $CD4^+$ and $CD8^+$ T cells increased in the pMB and BCG-treated mice and that there was a higher frequency of Ag85B secreting T cells, further supporting that vaccination with the pMB induced Th1 response in mice. In addition, $CD4^+$ and $CD8^+$ T cells remain the cornerstone of protective immunity against M.tb. Our data are consistent with previous findings that vaccination with a DNA vaccine encoding a fusion protein of HSP65 and human IL-2 significantly increases the frequency of peripheral blood $CD4^+$ and $CD8^+$ T cells and inhibits M.tb replication in mice (Changhong et al., 2009). At present, many studies have centered on the identification of the protective antigens of M.tb for developing DNA vaccines (Jonge et al., 2009; Bertholet et al., 2008). We found that the protective effect of pMB vaccination was similar to that of BCG, but significantly higher than that of vaccination with p85B in mice. Our results are in agreement with previous findings and further support the notion that vaccination with a DNA vaccine that expresses multiple antigens improves the protective effect against M.tb infection (Brun et al., 2008; Derrick et al., 2004; Li et al., 2006). So, vaccination with a DNA vaccine for multiple antigens is better to induce protective responses in mice.

In summary, results of immune response and protection mice of this study showed the group inoculated with pMB provided strong immune response and remarkable protection rate. However, if pMB plasmid would be a novel vaccine candidate for the prevention of TB, will depend on further study in future.

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