Immune Responses and Protective Efficacy Induced by 85B Antigen and Early Secreted Antigenic Target-6 kDa Antigen Fusion Protein Secreted by Recombinant Bacille Calmette-Guérin

Changhong SHI1*, Xiaowu WANG1, Hai ZHANG1, Zhikai XU2, Yuan LI2, and Lintian YUAN1

1 Laboratory Animal Research Center, Fourth Military Medical University, Xi’an 710033, China
2 Department of Microbiology, Fourth Military Medical University, Xi’an 710033, China

Abstract In an attempt to improve immune responses and protective efficacy, we constructed two recombinant bacille Calmette-Guérin (rBCG) strains expressing an 85B antigen (Ag85B) and early secreted antigenic target-6 kDa antigen (ESAT6) of Mycobacterium tuberculosis (MTB) fusion protein. Both rBCG strains have the same protein insertion but in a different order (Ag85B-ESAT6 and ESAT6-Ag85B). The cultured supernatant of rBCG strains and the sera from the mice immunized with the fusion protein Ag85B-ESAT6 or ESAT6-Ag85B formed a band with a fraction size of 37 kDa, equivalent to the sum of Ag85B and ESAT6. Six weeks after BALB/c mice were immunized with BCG or rBCG, spleen lymphocytes showed significant proliferation in response to culture filtrate protein of MTB. Compared with the BCG group, mice vaccinated with rBCG elicited a high level increase of immunoglobulin G antibodies to culture filtrate protein in the serum. The γ-interferon levels in the lymphocyte culture medium supernatants increased remarkably in the rBCG1 group, significantly higher than that of the BCG immunized group (P<0.05). Four weeks after vaccination, mice were infected with M. tuberculosis H37Rv and a dramatic reduction in the numbers of MTB colony forming units in the spleens and lungs was observed in the two rBCG immunization groups. Although these rBCG strains were more immunogenic, their protective effect was comparable to the classical BCG strain, and there were no significant differences between two rBCG groups (P>0.05).

Key words Mycobacterium tuberculosis; vaccine; Ag85B; ESAT6; BCG

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*Corresponding author: Tel, 86-29-84774787; Fax, 86-29-83291025; E-mail, changhong@fmmu.edu.cn

Tuberculosis (TB) is a global public health problem. Recent estimates from the World Health Organization indicate that there are approximately eight million new cases and three million deaths annually [1]. Factors such as multi-drug resistant strains, co-infection with HIV, and increasing mobility of population, have aggravated the situation. Bacille Calmette-Guérin (BCG) is the only vaccine used against TB worldwide, but it has variable protective efficacies, ranging from 0% to 80% in different field trials. Restoration of genes lost during the original BCG attenuation can enhance the ability of a recombinant strain to protect against Mycobacterium tuberculosis (MTB). Recombinant BCG (rBCG) is obtained by inserting an exogenous gene into BCG. This recombinant plasmid can express exogenous antigens, depending on the replication of BCG in the host, and induce specific humoral and cellular immune responses [2,3]. Several different types of rBCG vaccines against TB are being developed, such as BCG complemented with the complete RD1 region [4] and BCG complemented with listeriolysin [5] or Th1-type cytokines [6]. These BCG strains are all genetically modified BCG, with better protective effects against MTB infection, compared with conventional BCG. The rBCG over-expressing antigen 85B (Ag85B) of MTB is shown to improve protection in guinea pigs over that of BCG [7], indicating that an effective TB vaccine can be based on this restoration strategy.

However, further research has found that the protec-
tion afforded by rBCG expressing a single dominant antigen was only slightly superior to that of classical BCG. It may be a reasonable vaccine strategy to construct an rBCG expressing a fusion protein to provide stronger protection against TB, such as interleukin-2 and early secreted antigenic target-6 kDa antigen (ESAT6) of MTB [8], or different protective antigens from the culture filtrate protein (CFP) of MTB [2].

Ag85B and ESAT6 as a fusion protein had been previously shown, by the team of Peter ANDERSEN, to induce a protective immunity higher than a mixture of these two proteins not in fusion [9]. So in this study, we constructed two rBCG strains with the same protein insertion in a different order (Ag85B-ESAT6 and ESAT6-Ag85B), then compared their immune responses in mice induced by these two rBCG strains, and their protection against MTB infection.

Materials and Methods

Materials

The pGEM-T Easy vector was purchased from Promega (Madison, USA). 7H9 (broth), 7H10 (agar) medium and albumin-dextrose-catalase (ADC) enrichment were purchased from Difco (Detroit, USA). The mouse interferon (IFN)-γ enzyme-linked immunosorbent assay kit was purchased from Jinmei (Shenzhen, China). The goat anti-mouse immunoglobulin (Ig)G-conjugated horseradish peroxidase was purchased from Sino-American Biotechnology (Luoyan, China). The sera from mice immunized with the fusion protein Ag85B-ESAT6 or ESAT6-Ag85B, and CFP (final concentration 25 mg/L) of MTB were prepared in our laboratory. Mice were immunized three times at two-week intervals by subcutaneous injection with 0.6 ml of 50% (V/V) mixture of fusion protein Ag85B-ESAT6 or ESAT6-Ag85B (50 µg/ml in water) and incomplete Freund’s adjuvant (Sigma-Aldrich, St. Louis, USA). Sera were collected 10 d after the last immunization and used for Western blot analysis. The BCG vaccine strain was obtained from the Lanzhou Bioethical Production Institute (Lanzhou, China). Pathogen-free BALB/c female mice were provided by the Animal Center of the Fourth Military Medical University (Xi’an, China). The mice were 6–8 weeks old and maintained in barrier system. All animals had free access to water and were fed standard mouse chow.

Construction and screening of rBCG

The recombinant Escherichia coli-BCG shuttle plasmid Ag85B-pDE22-ESAT6 expressing the fusion protein Ag85B-ESAT6 was constructed as follows. The genes of Ag85B and ESAT6 were amplified from the genome of MTB H37Rv by polymerase chain reaction (PCR). The primers, synthesized by AuGCT Biotechnology (Beijing, China), for Ag85B were A1F and A1R. The sequence of A1F was 5′-GGATATCAAGCTTGCCATGACAGAGCAGCTGG-3′ (HindIII site underlined) and that of A1R was 5′-GGGATTCATCGATCTATGCGAACAATCCCCAGTGAC-3′ (EcoRI site underlined). The Ag85B-ESAT6 fusion construct was inserted at the unique HindIII site, introduced by primers A1R and E1F. The recombinant E. coli-BCG shuttle plasmid, ESAT6-pDE22-Ag85B, expressing the fusion protein ESAT6-Ag85B, was constructed as follows. Primers for ESAT6 were E2F, 5′-GGGATTCATGACAGAGCAGCTGG-3′ (BamHI site underlined) and E2R, 5′-GAAAGCTTGGCAACATCCCCAGTGAC-3′ (HindIII site underlined). Those for Ag85B were A2F, 5′-GCAAGCTTGGCAACATCCCCAGTGAC-3′ (HindIII site underlined) and A2R, 5′-GGGATTCATCGATCTATGCGAACAATCCCCAGTGAC-3′ (EcoRI site underlined). The ESAT6-Ag85B fusion construct was also inserted at the unique HindIII site, introduced by primers E2R and A2F. All DNA sequences were confirmed by sequencing (AuGCT Biotechnology). The genes coding for Ag85B and ESAT6 were inserted into the corresponding sites of the multiple cloning sites region of E. coli-BCG shuttle plasmid pDE22 (containing Hsp60 promoter and α-ss signal sequences).

The BCG vaccine and MTB H37Rv strains were cultured in 7H9 medium (containing 100 g/L ADC and 0.5 g/L Tween-80) at 37 °C for 3 weeks. The bacterium were harvested and stored at −20 °C. The concentrate was serially diluted with 7H9 medium, and inoculated in Lowenstein-Jensen medium, then grown at 37 °C for 2 weeks. Competent BCG cells were produced by growth in 7H9 medium to the logarithmic phase, incubated for 1h in an ice bath, then washed three times with 10% glycerin. Purified Ag85B-pDE22-ESAT6 or ESAT6-pDE22-Ag85B plasmids were transfected into 5×10^6 competent BCG cells by electroporation in a 2 mm gap cuvette at 2.5 kV voltage, 25 µF capacitance and 1000 Ω resistance. The transformants were screened on 7H10 agar plates containing ADC and hygromycin. The positive clones were amplified and confirmed by PCR and the culture supernatants were harvested, concentrated by PEG4000 and dialyzed against phosphate-buffered saline (PBS) for subsequent sodium
dodecyl sulfate-polyacrylamide gel electrophoresis analyses. Western blotting was carried out as follows. Sera from mice immunized with the fusion protein Ag85B-ESAT6 or ESAT6-Ag85B were used as the first antibody. The second antibody was goat anti-mouse IgG-conjugated horseradish peroxidase. The substrate solution contained ortho-phenylenediamine (1 mg/ml) and H$_2$O$_2$ (30%, V/V) in PBS. The rBCG strain expressing the Ag85B-ESAT6 fusion protein was named rBCG1 and that expressing the ESAT6-Ag85B fusion protein was named rBCG2.

**Animal immunizations**

Forty BALB/c mice were divided randomly into four groups of 10 mice. Three groups were injected subcutaneously with 10$^6$ colony forming unit (CFU) of rBCG1, rBCG2 or BCG in a volume of 100 µl for each mouse. The fourth group was inoculated with normal saline as a control. After 6 weeks, five mice in each group were used for the immunological assays. The remaining five mice in each group were used for a virulent strain infection experiment.

**Specific lymphocyte proliferation assay**

Six weeks after immunization, four groups of five mice each were killed and their spleens removed aseptically. Single cell suspension was obtained by forcing the spleen through a 200-gauge stainless steel mesh and prepared in RPMI-1640 medium containing 10% fetal calf serum (FCS), 2 mM glutamine, 50 µg/ml streptomycin and 100 U/ml penicillin. The erythrocytes were lysed by incubation in a solution of 0.84% ammonium chloride in distilled water. Lymphocytes were counted and used in the following assay. Two hundred microliters of 5×10$^5$ lymphocytes/ml was seeded in 96-well plates, and cultured in RPMI-1640 medium with 10% FCS. The cells were treated with CFP (25 mg/L final concentration) of MTB in the treated group [10]. Preliminary titration experiments had shown that a threshold concentration for stimulating a maximum specific response was between 25 and 50 µg/ml for CFP of MTB. As the concentration of 25 µg/ml was the minimum unit that provided a maximum response to CFP, it was used throughout the experiment. The cells were incubated at 37 ºC for 68 h under an atmosphere of 5% CO$_2$. Twenty microliters of MTT was added to each well at a concentration of 5 mg/ml. After incubation for 4 h, the MTT was removed and replaced with 150 µl dimethyl sulfoxide, and was incubated for 10 min at 37 ºC until the crystals were dissolved. CoA (10 µg/ml) (Sigma-Aldrich) served as the positive control. The optical density value of each well was measured using a microculture plate reader, with a test wavelength of 490 nm. Proliferation was expressed as the result of the following equation:

\[
\text{Stimulation index (SI)} = \frac{A_{490\text{ (treated)}}}{A_{490\text{ (control)}}}
\]

**IFN-γ content assay**

Eight hundreds of spleen lymphocytes (5×10$^6$ cells/ml) from immunized mice were seeded in a 24-well plate, cultured in RPMI 1640 medium with 10% FCS and incubated with MTB CFP 100 µl/well (25 µg/ml) at 37 ºC for 72 h in an atmosphere of 5% CO$_2$. The supernatant was harvested after centrifugation at 2500 g for 5 min, and stored at -20 ºC. The IFN-γ levels in the lymphocyte culture medium supernatants were analyzed using a commercial enzyme-linked immunosorbent assay kit (Jingmei) according to the manufacturer’s instructions. All samples were diluted from 10 to 1000 folds, to scale the concentrations to fall within the detectable range. The measurements were then repeated at different dilutions to confirm the validity of the analyses. The data are shown as mean±SD of five mice in each group.

**Antibody level evaluation in sera of vaccinated mice**

Sera were collected from the immunized mice in each group. Microtiter plates were coated overnight with 100 µl CFP (25 µg/ml) at 4 ºC, then blocked with 1% bovine serum albumin in PBS. Serum samples were diluted to appropriate concentrations (10–30-fold dilution) and incubated for 2 h at 37 ºC. Horseradish peroxidase-conjugated goat anti-mouse IgG was added for detection of antibodies and the substrate solution containing ortho-phenylenediamine (1 mg/ml) and H$_2$O$_2$ (30%, V/V) in PBS. Antibody titers were calculated by linear regression analysis, plotting dilution versus $A_{490}$.

**Protection against MTB infection**

Six weeks after immunization, the mice were infected with the MTB virulent strain H37Rv through the tail vein at a dose of 10$^4$ CFU/mouse. The immunized mice were killed at 28 d post-infection and spleens and lungs harvested and homogenized in 3 ml of Middlebrook 7H10 agar plates medium (Difeo) supplemented with PBS containing 0.05% (V/V) Tween-80 for CFU counting.

**Statistical analysis**

The statistical significance of the difference was estimated by Student’s t-test. The differences were considered statistically significant when $P$ values were less than 0.05.
Results

Screening of the positive clone containing rBCG

Screening by hygromycin produced a total of seven colonies on the 7H10 plate. PCR was carried out to select the recombinant clones containing Ag85B-pDE22-East6 and ESAT6-pDE22-Ag85G. The acquired 1168 bp fragment was equal in fraction size to the sum of Ag85B and ESAT6. Western blot analysis of the rBCG supernatant revealed an expression band with a relative fraction size of 37 kDa (Fig. 1). As the fraction size of Ag85B was 31 kDa and ESAT6 was 6 kDa, this product was considered to be the fusion protein of Ag85B and ESAT6. The cultured supernatant of the two screened rBCG strains and the sera from the mice immunized with the fusion protein Ag85B-ESAT6 or ESAT6-Ag85B formed a band with a relative fraction size of 37 kDa, equivalent to the sum of Ag85B and ESAT6. As a result of deletion of the ESAT6 gene, BCG expressed only Ag85B and was unable to express ESAT6. Western blot analysis of the sera from the mice immunized with fusion protein and the supernatants of BCG formed a band with a relative fraction size of 31 kDa. However, the levels of expression of fusion proteins Ag85B-ESAT6 and ESAT6-Ag85B in the supernatants of rBCG were significantly different.

Growth curve of rBCG

The two rBCG strains that secreted the Ag85B-ESAT6 and ESAT6-Ag85B fusion proteins and the conventional BCG strain were cultured and quantitated. As shown in Fig. 2, there were three BCG logarithmic growth periods after 15 d, and a platform period after 45 d. The two rBCG strains showed no significant differences in proliferation characteristics, compared with the BCG strain.

Antigen-specific spleen lymphocyte proliferation

Fig. 3 shows the results of antigen-specific lymphocyte proliferation responses to CFP of MTB. Compared with the saline group, high level SIs were induced by the two rBCG strains. However, the SIs of the two rBCG groups and the conventional BCG group showed no apparent differences. ConA served as a positive control and

Fig. 1  Expression of fusion protein Ag85B-ESAT6 or ESAT6-Ag85B in the supernatants of classical BCG (A) and rBCG (B) by Western blot analysis

1, using anti-fusion protein Ag85B-ESAT6 antibodies; 2, using anti-fusion protein ESAT6-Ag85B antibodies; 3,4, form rBCG1 and rBCG2 using antibodies recognizing Ag85B and ESAT6 fusion proteins.

Fig. 2  Growth curve of the recombinant bacille Calmette-Guérin (rBCG) strains

The two rBCGs and BCG strains entered the logarithmic growth period after culturing 15 d and a platform period after 45 d. The two rBCGs had no significant difference in proliferation characteristics compared with classical BCG.

Fig. 3  Spleen lymphocyte proliferative responses to stimulation by culture filtrate protein of Mycobacterium tuberculosis (n=5)
The spleen lymphocytes of immunized mice were cultured with culture filtrate protein (CFP; 25 µg/ml) of M. tuberculosis. Proliferative response was expressed as the result of the stimulation index (SI). Data represent the mean and standard error of five mice per group. BCG, bacille Calmette-Guérin; rBCG, recombinant bacille Calmette-Guérin.
showed an SI of 7.10±0.40.

IFN-γ levels in spleen lymphocytes

After the spleen lymphocytes of the immunized mice were stimulated with CFP of *M. tuberculosis*, the IFN-γ levels in the lymphocyte culture medium supernatants were analyzed. IFN-γ levels increased remarkably in the rBCG1 group and the rBCG2 group, above that in the saline control group, and significantly higher than that of the BCG immunized group (*P*<0.05) (Fig. 4).

**Antibody response**

The MTB H37Rv CFP serum antibody levels of five mice in each group are shown in Fig. 5. Animals vaccinated with BCG and rBCG elicited specific antibodies against CFP. Compared with the BCG group, there was a large increase in IgG antibody level for both rBCG1 and rBCG2 group, however no significant difference was observed between the two rBCG groups.

**Resistance against MTB infection in immunized mice**

Four weeks after mice were infected with MTB H37Rv, the spleen and lung bacterial loads of the rBCG mice were determined, and are shown in Table 1. Both of the rBCG vaccines could effectively resist MTB infection, and reduced the splenic bacterial load compared with the saline group (*P*<0.05). But the protective efficacy of the rBCG vaccine did not exceed that of the classical BCG vaccine, and there was no significant difference between rBCG and BCG groups (*P*<0.05).

**Discussion**

Despite its many shortcomings, BCG is an effective vaccine used widely for prevention of TB. Given shortly after birth, BCG reduces the incidence of childhood TB effectively, but has little or no effect (protective efficacy ranges from 0% to 80%) on the predominantly adult disease responsible for the current global emergency [11]. With two million TB-related deaths worldwide each year, there is a pressing need for improved vaccines. Therefore, efforts to develop new TB vaccines should use the documented advantages afforded by BCG. In new rBCG strains, a target gene is introduced into a BCG vector, or is cloned positionally to acquire high expression without influencing the survival and multiplication capacity of the strain [12]. Inoculation of this recombinant vaccine can provide...
a protection efficacy not only against the original bacteria, but also against the disease associated with the introduced genes. The key to constructing a TB vaccine with recombinant BCG techniques is determining which exogenous genes have been introduced into BCG. The optimal gene should increase strain immunogenicity and stimulate the immunologic memory, but not change the low virulence protective capacity of BCG.

The human body resists MTB infection mainly by cellular immune response, and the MTB protein that can induce this immune response occurs in the early culture stage of the bacteria, namely during its logarithmic growth. In the numerous secreted MTB proteins, Ag85B can induce a Th1-type cellular immune response in animal models, which has a resistance against MTB reinfection that is similar to that of BCG [13]. ESAT6 is a protein secreted in the cultures of the MTB virulent strains. Its encoding gene exists in the MTB virulent strains, but not in the attenuated strains. ESAT6 is one of the main target antigens recognized by T cells in the recall immune response of the MTB-infected human or animal [14]. Thus, ESAT6 is a potential component candidate for a new vaccine against MTB. After studying the immune response of univalent and multivalent Bacillus tuberculosis DNA vaccines, it was claimed that a multivalent combination DNA vaccine had greater protective efficacy than BCG [15]. Thus, the multivalent vaccine containing multiple MTB immune response-related antigens is a more effective vaccine. Vaccination with the Ag85B-ESAT6 fusion protein in adjuvant has been shown to induce a protective immune response higher than a mixture of these two proteins not in fusion by the team of Peter ANDERSEN [9,16]. In this study, the BCG recombinant fusion protein Ag85B-ESAT6 served as target antigen to gain an improved immune response over that induced by a single component.

The protective effect of the rBCG strain over-expressing Ag85B in guinea pigs was significantly higher than that of the BCG vaccine [7], suggesting that the rBCG strain possesses superior immunization potential. The virulence of the rBCG strain expressing ESAT6 was not significantly higher than that of the BCG immunized group, as determined by survival time, bacterial number and pulmonary pathology section. This rBCG could induce an improved cellular immunologic response over that of BCG, despite the finding that its protection in mice did not exceed that afforded by BCG [9]. In this study, we successfully cloned an Ag85B and ESAT6 fusion gene with the potential to protect against tuberculosis. These two rBCG strains we obtained possess proliferation characteristics similar to the conventional BCG strain, entering the logarithmic growth period after 15 d and the platform period after 45 d. The introduction of the fusion gene did not influence the growth of rBCG.

Many studies have attempted to develop a TB vaccine using rBCG techniques. These consist mainly of recombining cytokines [17,18] or the dominant antigen for cell-mediated immunity (such as ESAT6) [3] and the major target for memory T cells (such as Ag85B) [7] into BCG. Vaccination with these rBCG strains has been shown to be an effective vaccine strategy, and even some rBCG strains afforded protective efficacy over the standard BCG vaccine. The above-mentioned research suggests that a gene or subunit vaccine with multi-epitopes can induce stronger cellular immunological responses and protection than single epitope vaccines, with a single subdominant epitope of the antigen, which might be poorly recognized during natural infection [19]. The study of Palendira et al. showed that vaccination of mice with an rBCG secreting high levels of Ag85B-ESAT6 induced a protective immunity against aerosol MTB higher than a mixture of these two proteins not in fusion, as expression of the single components did not significantly increase the protective efficacy of rBCG [2]. Six weeks post-immunization with rBCG expressing Ag85B-ESAT6 or ESAT6-Ag85B in this study, higher serum antibody responding to CFP of MTB H37Rv was observed, and the level of induced IFN-γ was significantly higher than those induced by the BCG or saline groups. However, there was no apparent difference between the two rBCG strains. This shows that the orders of Ag85B and ESAT6 in recombinant BCG vector have little influence on the biological characters of rBCG.

Even though the two rBCG strains used in our study could effectively induce Th1 cell immune response, especially with respect to the IFN-γ levels in spleen lymphocytes, their protection in mice did not exceed that of the current BCG after MTB challenge, indicating that the protective effect was not compliant with the cellular immune response. This suggests that the immune response induced by a single expression strategy is limited, and that an effective TB vaccine should be complex, with multiple epitopes, and affording efficacy through multiple routes. Ag85B and ESAT6 proteins, although preferable for the measurement of specific responses, were difficult to obtain. Therefore, the CFP of MTB was used as the antigen in all assays, which could change the level of antigen-specific response and affect the interpretation of the results. Our future research should use the purified Ag85B and ESAT6 proteins to determine whether a response specific to these antigens was actually induced by rBCG.

Pym et al. pointed out that an increase in BCG viru-
lence was the result of the cooperative action of multiple genes, and that only the introduction of the complete RD1 domain into BCG could increase its virulence [20]. Introduction of the ESAT6 gene into BCG would not only change the low virulence of BCG, but also be an optimal candidate vaccine for TB. Note that a safety evaluation on rBCG was not carried out in this study because Ag85B and ESAT6 have been used for rBCG, and there are no published reports of an increased virulence.

In conclusion, two rBCG strains expressing a fusion of Ag85B and ESAT6 with the same protein insertion and different order have been successfully constructed. They could induce high level humoral and cellular immune responses and reduce bacterial loads effectively within the lung and spleen. The order of Ag85B and ESAT6 in the fusion gene had little influence on the immune responses (such as lymphocyte proliferation responses, IFN-γ production, antibody level, and protective efficacy) in mice vaccinated with the rBCG strains. The rBCG strains used in this trial did not increase efficacy over classical BCG. The following methods might be considered for improvement of immunogenicity and protective efficacy: (1) choosing a more susceptible animal such as the guinea pig to study the protective efficacy of MTB; and (2) using an E. coli-BCG shuttle vector with a high-efficiency promoter to study antigen expression.

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