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Immune response induced by recombinant *Mycobacterium bovis* BCG expressing ROP2 gene of *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is an obligate intracellular parasite, capable of infecting a variety of mammals and birds. Development of vaccine against *T. gondii* would be of great medical and veterinary value. In this study, the DNA sequence encoding ROP2 from *T. gondii* was cloned into the muticopy mycobacterial expression vector, pMV262, under the control of the Bacillus Calmette–Guerin (BCG) *hsp*60 promoter, and electroporated into BCG. Following selection of kanamycin, the recombinant BCG/pMV262-ROP2 was constructed and the expression of ROP2 was confirmed by Western blotting. The BALB/c mice inoculated with the BCG/pMV262-ROP2 developed specific immune responses against ROP2 protein, and there was an obvious delay in the mortality curve than the control ($P<0.05$). These results indicated that *M. bovis* BCG is an adequate vector to express and present antigens of *T. gondii*, and it may be used to further study the induction of protective immunity in other animals.

Keywords: *Toxoplasma gondii*; BCG; ROP2; Immune

1. Introduction

Toxoplasma gondii, an obligate intracellular protozoan parasite, infects multiple vertebrate species and invades a broad variety of nucleated host cells. Infection with *T. gondii* is common, one-third of the human population in the world is estimated to be infected with the parasite, and adults are usually asymptomatic, but *T. gondii* can cause serious clinical symptoms (encephalitis, retino-choroiditis, abortion, abnormal embryo, even fetal death) in certain conditions, such as AIDS sufferers, pregnant women and children [1–3]. In addition, toxoplasmosis can cause considerable economic loss in the farming industry [4,5]. Thus, development of vaccine against *T. gondii* would be of great medical and veterinary value. The act that a single infection with any strain of *T. gondii* can lead to lifelong immunity against re-infection in immunocompetent individuals denotes that the development of an effective vaccine against toxoplasmosis is a realistic goal [6,7].

Protection against toxoplasmosis is largely mediated by cellular immunity, and this type of immunity can best be achieved by immunization with a live vaccine [8]. Such live vaccines are believed to better deliver the proteins to antigen-presenting cells, especially when the vaccines are *in vivo*-replicating intracellular microorganisms [9]. In several instances, live attenuated *T. gondii* strains have been shown to provide protection against this disease [10]. However, such strains are poorly characterized at the genetic level, carry the inherent risk of reverse to virulence and are impractical for human use. Therefore, much attention has been focused on the identification of protective antigens and the use of modern technology to deliver them to the host. ROP2 (rhoptry protein 2) is secreted by the rhoptry and expressed in tachyzoite, bradyzoite and cyst stages of *T. gondii*, which is involved in

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invasion of host cells, making it a promising candidate antigen for development of subunit vaccine or genetically engineering vaccine [8,11]. It has been shown that vaccination with *T. gondii* ROP2 can increase the host survival rates after a lethal infection [12,13].

Bacille Calmette–Guerin (BCG), an attenuated *Mycobacterium bovis* strain, has been used extensively over decades as a live vaccine against tuberculosis without significant adverse effects. Furthermore, BCG possesses intrinsic adjuvant properties, especially for the development of cell-mediated immunity, which has recently led to the use of BCG as a foreign antigen delivery system [14,15]. Both cellular and humoral immune responses against a number of foreign antigens have been observed in mice after vaccination with recombinant BCG. Some of these responses were shown to provide protection against challenge with the corresponding pathogens [16–18]. Since both *M. bovis* BCG and *T. gondii* are intracellular microorganisms, the recombinant BCG would be particularly appropriate for the development of a vaccine against *T. gondii*. In this study, we report construction of recombinant *M. bovis* BCG expressing ROP2 from *T. gondii* and BCG/pMV262-ROP2 could induce specific immune responses against ROP2 protein in mice.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli strain DH5α was grown in LB medium at 37 °C with shaking (100 rpm). *M. bovis* BCG, sub-strain Pasteur, was grown without shaking in culture flasks of 25 cm^2 at 37 °C in Middlebrook 7H9 liquid medium supplemented with 10% albumin-dextrose complex (ADC) enrichment (Difco). Both *E. coli* and mycobacteria were supplemented as required with 20 μg/ml kanamycin (Sigma).

2.2. Cloning of the ROP2 gene in the mycobacterial expression vectors

The mycobacterial expression plasmids pMV262 contains a multi-cloning site, origins of replication in both *E. coli* and mycobacteria, which is an extrachromosomal mycobacterial vector containing the mycobacterial heat shock protein 60 (*hsp*60) promoter and a kanamycin resistance gene [19].

For cloning, the 1224-bp fragment encoding complete ROP2 (GenBank accession no. Z36906) was amplified by polymerase chain reaction (PCR) from *T. gondii* (RH strain) genome using the oligonucleotides with the following sequences: 5′-AT*GAATTC*-GCGGCGGCATCTAGATTCTTTAGG-3′ and 5′-AT*AAGCTT*-TCATGCCGGTTCTCCATCAGTTTG-3′, and containing a *Eco*RI and a *Hind*III restriction sites (italic), respectively. PCR reactions were carried out in a thermocycler using 30 cycles of 1 min of denaturation (94 °C), 45 s of annealing (63 °C), and 2 min of extension (72 °C) after incubation at 94 °C for 5 min. The PCR product was digested with *Eco*RI and *Hind*III and then inserted into the *Eco*RI and *Hind*III sites of the *E. coli*-BCG shuttle expression vector pMV262, yielding pMV262-ROP2.

2.3. Construction of recombinant M. bovis BCG

The cultivation, transformation and inducing expression of *mycobacteria bovis* BCG were carried out as described previously [20]. Briefly, BCG was grown to an optical density of 600 nm (OD_{600}) of 0.6, washed twice at 4000 rpm for 10 min, and resuspended in 1/10 of original volume in 10% glycerol. Two hundred microlitres of BCG were mixed with plasmid DNA (1–2 μg) in a pre-chilled 0.2 cm electroporation cuvette (BTX). BCG was transformed with the recombinant plasmids using a Gene Pulser (BTX) at 2.5 kV, 1000 Ω and 25 μF. After transformation, 800 μl of culture medium was added to the mixture followed by incubation at 37 °C for 24 h. The bacteria were then plated in Middlebrook 7H9 medium supplemented with 20 μg/ml of kanamycin.

After 3 weeks, individual colonies were transferred to liquid medium for further verification of recombinant protein expression.

2.4. Expression of ROP2 in BCG

Expression of ROP2 in BCG was demonstrated using Western blotting (WB) as described previously [21]. Briefly, Recombinant BCG was grown to the optical density of 600 nm (OD_{600}) of 0.6–1.0 (about 16–20 days), then induced at 45 °C for 2 h. Ten milliliters of the recombinant mycobacterial culture were washed once in PBS (phosphate-buffered saline), followed by suspension in 100 μl of the lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 2 mM PMSF). Cells were disrupted in the presence of 50 mg of glass beads (0.1 mm diameter), applying three 30 s pulses in a mini-beadbeater. Subsequently 33 μl of 4 × concentrated sample buffer (100 mM Tris–HCl, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added and the cell lysates were analyzed by SDS-PAGE.

For Western blotting analysis after electrophoresis, non-stained gels were transferred to the nitrocellulose membrane (Bio-Rad). The membranes were incubated with blocking reagent (5% milk in PBS) at room temperature and subsequently reacted with rabbit anti-*T. gondii* polyclonal primary antibody diluted in 10 mM Tris–HCl (pH 8.0), 0.15 M NaCl and 0.05% Tween-20. After washing out the primary antibody, the membranes were processed with the goat-anti-mouse IgG labeled by alkaline phosphatase (Sigma). Reacting bands were detected by BCIP/NBT (5-bromo–4-chloro–3-indolyl phosphate/nitro blue tetrazolium).

2.5. Immunization and challenge infection

Four-week-old BALB/c mice were provided by Center of Laboratory Animal in Shandong Province and all animal experiments were approved by Center of Laboratory Animal in Shandong Province.

Eighty BALB/c mice were randomly divided into four groups, twenty in each group. All mice were inoculated by subcutaneous route. Group 1 was immunized with 0.1 ml PBS; group 2 was immunized 0.1 ml wild BCG/pMV262 (10^7 cfu/ml), substrain Pasteur; Group 3 was immunized with 0.1 ml BCG/pMV262-ROP2 (10^7 cfu/ml); group 4 was immunized twice

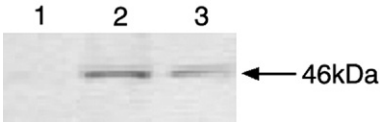

Fig. 1. Expression of ROP2 in BCG/pMV262-ROP2 analyzed by Western blotting. Line 1, Negative control (BCG/pMV262); Line 2, Recombinant ROP2 expressed in *E. coli*; Line 3, BCG/pMV262-ROP2.

with 0.1 ml BCG/pMV262-ROP2 (10^7 cfu/ml) at 4 weekly intervals.

Two weeks post-second immunization, mice were challenged intraperitoneally with 500 *T. gondii* (RH virulent strain) trophozoites, and their survivals were monitored twice daily.

2.6. Immune response induced by BCG/pMV262-ROP2 in mice

Specific antibodies induced by BCG/pMV262-ROP2 were evaluated by Western blotting and ELISA. Mice were bled from the tail-vein at different time points. Sera were individually tested by immunoblot analysis for the presence of antibodies reactive against ROP2 present in *T. gondii* lysates. *T. gondii* lysates were prepared by three freeze-thaw cycles of tachyzoites of the RH strain, then suspended in SDS-PAGE loading buffer, followed by boiling at 100 °C for 5 min. Lysates were separated by SDS-PAGE and then transferred onto nitrocellulose membrane. Western blotting was performed as mentioned above. ELISA was performed according to described previously [22].

For evaluation of cellular immunity, splenocytes from inoculated mice were isolated and used to detect production of IL-2 and IFN-γ by ELISA [18], and T lymphocyte subsets by flow cytometry. Briefly, mice were killed at 14 days post-third immunization and their spleens were removed aseptically. Spleen single cell suspension was prepared as described previously [9]. The splenocytes were finally counted at 10^7 cells/ml in RPMI-1640 (Difco) supplemented with 10% foetal bovine serum (Gibco), and incubated for 68 h with 80 μg recombinant ROP2 expressed in *E. coli*, whose purity could reach 96.5%, which was purified by Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) affinity chromatograph [23]. After incubation, supernatants were collected and the production of IL-2 and IFN-γ was determined using the ELISA kit (Jingmei Biotech Co., Ltd. China) following manufacturer's instructions.

2.7. Statistical analysis

Results of serological assays, production of cytokines and survival days of immunized mice against *T. gondii* challenge were compared using analysis of variance (ANOVA) and *t*-test using the SPSS software. A *P*-value of less than 0.05 was considered different.

3. Results

3.1. Plasmids construction and expression of ROP2 in BCG

A PCR product of 1 224 bp, corresponding to ROP2 coding sequence, was obtained, digested with the appropriate restriction enzymes and cloned in the *E. coli*-BCG shuttle expression vectors pMV262, generating pMV262-ROP2. BCG was transformed with the recombinant plasmids pMV262-ROP2 by electroporation, and induced at 45 °C for 2 h for expression of ROP2. The production of ROP2 was assessed by immunoblot analysis, using polyclonal mouse antibodies. Special immunoreactive proteins were detected in the cell lysates of BCG/pMV262-ROP2, whereas they were absent in BCG/pMV262 (Fig. 1). These results indicated that ROP2 was successfully expressed in BCG/pMV262-ROP2.

3.2. Humoral immune response induced by BCG/pMV262-ROP2

Three groups of BALB/c mice were vaccinated with PBS, BCG/pMV262, BCG/pMV262-ROP2 once and the other group was boosted with BCG/pMV262-ROP2 of the same dose on days 28. Blood samples were collected every 14 days. Total antibodies in BCG/pMV262-ROP2-inoculated mice were detectable at 2 weeks, and stable increasing during the first 4 weeks post immunization. When the mice received a boost of inoculation, the humoral immune level had been induced much higher than received a single inoculation (Fig. 2). The immune sera were also analyzed by Western blot, indicating that the immune sera could specifically recognize the native antigen extracted from *T. gondii* tachyzoites and the recombinant ROP2 expressed by BCG/pMV262-ROP2 (data not shown).

3.2.1. Cellular immune responses induced by BCG/pMV262-ROP2

Cellular immune responses were evaluated by measuring IL-2 and IFN-γ in splenocytes from mice inoculated with BCG/pMV262-ROP2. Splenocytes from inoculated mice were stimulated with the recombinant ROP2. The supernatants were assayed in triplicate to verify the production of IL-2 and IFN-γ. The production of IFN-γ and IL-2 was detectable in BCG/pMV262-ROP2 and BCG/pMV262 inoculated mice, and its level had been induced much higher than received a single inoculation when the mice received a boost of inoculation with BCG/pMV262-ROP2. As expected, no significant production of IFN-γ and IL-2 was detected in PBS inoculated mice (Fig. 3). These results indicated that the BCG/pMV262-ROP2 expressing ROP2 protein of *T. gondii* could induce cellular immune responses in mice.

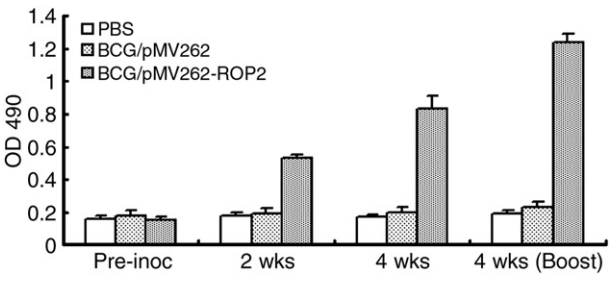

Fig. 2. Humoral immune response induced by BCG/pMV262-ROP2 in mice, which was detected by ELISA. Results were reported as the arithmetic means± standard errors (n=10 mice per group).

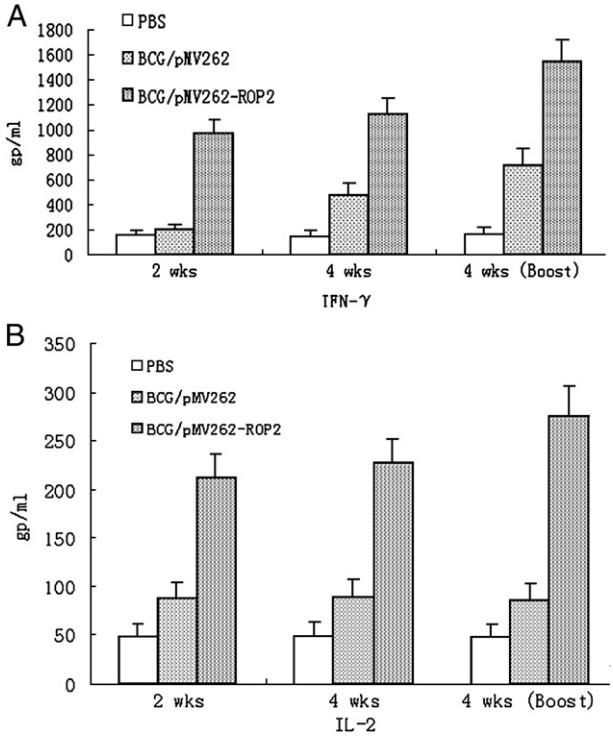

Fig. 3. Production of IFN-γ and IL-2 in splenocytes from inoculated mice. Results were reported as the arithmetic means±standard errors ($n=10$ mice per group). A. Production of IFN-γ; B. Production of IL-2.

The percentage of $CD4^+$T cells from mice immunized with BCG/pMV262-ROP2 showed obvious increase compared with that of controls including BCG/pMV262 and PBS ($P<0.01$) but the percentage of $CD8^+$T cells was no obviously increased ($P>0.05$) (Fig. 4.).

3.3. Protective effect of mice immunized with BCG/pMV262-ROP2 against T. gondii challenge

To assess the direct protective potential of the recombinant BCG in mice, 10 BALB/c mice were immunized either with 10^6 BCG/pMV262-ROP2 or with the same amount of BCG/pMV262 and PBS. The animals were immunized twice with 4 weekly intervals. They were then challenged intraperitoneally with 500 *T. gondii* (RH virulent strain) trophozoites at 2 weeks after the second immunization. Compared to the mice immunized with the control PBS and BCG/pMV262, which all died within 8 and 9 days, there was an obvious delay in the mortality curve of the animals immunized with BCG/pMV262-ROP2 ($P<0.05$) (Fig. 5).

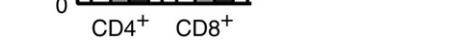

Fig. 4. Changes of $CD4^+$and $CD8^+$T cells in splenocytes from inoculated mice.

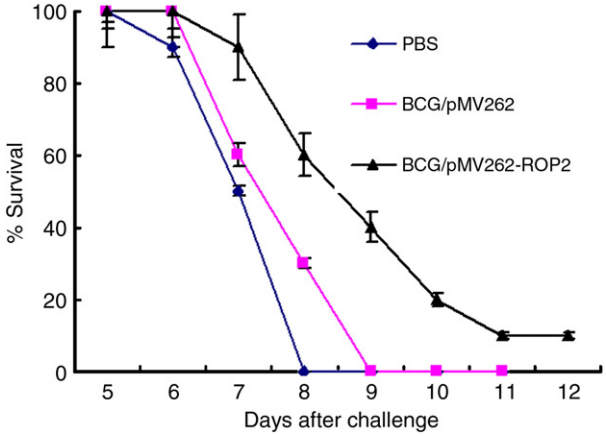

Fig. 5. Survival curves of mice immunized with BCG/pMV262-ROP2, BCG/pMV262 against *T. gondii* challenge. The challenge experiment was repeated three times and results were reported as the arithmetic means±standard errors ($n=10$ mice per group).

4. Discussions

BCG was used as live vector expressing ROP2 gene of *T. gondii* based on the following considerations. Firstly, BCG is an attenuated strain, which was derived from *M. bovis* and does not revert towards a virulent phenotype [24]. Secondly, it has been used for human vaccination against tuberculosis. Today more than 3 billion people have been vaccinated with BCG and the incidence of important side effects is very low [25]. Thirdly, BCG can be given at birth or any time thereafter, and can induce a good immune response in individuals who have previously been vaccinated with BCG. It is also inexpensive to produce and relatively thermostable [26]. Finally, it s a strong immunostimulant and has been used as an adjuvant in various immunization programs [27].

In this study, the DNA sequence encoding ROP2 from *T. gondii* was cloned into the muticopy mycobacterial expression vector, pMV262, under the control of the BCG *hsp*60 promoter, and electroporated into BCG. Following selection of kanamycin, the expression of ROP2 was confirmed by Western blotting. The immune assay demonstrated that the recombinant BCG expressing ROP2 could produce special humoral and cellular immune responses in mice, and immune level had been much higher than received a single inoculation when the mice received a boost of inoculation. There was an obvious delay in the mortality curve of the animals immunized with recombinant BCG producing ROP2.

Choice of protective antigens is the key to development of vaccine for *T. gondii*. The parasite replicates within a

specialized vacuole surrounded by the parasitophorous vacuole membrane (PVM). ROP2, which is localized to the PVM, is one of the proteins secreted from rhoptries during parasite invasion into host cells [28,29]. Only one antigen perhaps could not induce complete protection against this unicellular pathogen [30].

During *T. gondii* infection, the humoral responses are predominant in the earlier period, but the cellular responses are primary in the later or convalescence stage. It also has been demonstrated that heterologous expression in the mycobacterial cytoplasm induces predominantly cellular immunity, while antigens presented on the mycobacterial surface elicit higher humoral immune. In this study, BCG/pMV262-ROP2 inoculated mice developed both humoral and cellular immune against the parasite protein, indicating that the recombinant antigen was adequately presented to the mice's immune system, but did not induced complete protection against infection with *T. gondii* RH strain. The immune responses elicited against ROP2 usually differs in different mouse strains [21,31]. It is necessary that the immune response and protection of BCG/pMV262-ROP2 in other animal models be evaluated, for example C57BL/6 mice and C3H mice.

BCG is the most used of all vaccines. It is important to know whether or not recombinant BCG can induce good immune responses in individuals who have previously been vaccinated with BCG. In our study, successive immunization did not decrease the immune responses in mice, which is consistent with the previous report [32], demonstrating BCG is unlike other vectors, such as adenovirus vector producing antibodies against adenovirus and decreasing the immune responses when repetitve administration [33].

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