Construction of a recombinant vaccinia virus expressing *Babesia gibsoni* thrombospondin-related anonymous protein and evaluation of its immunogenicity in mice

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ABSTRACT

Previously, we have identified a gene encoding thrombospondin-related anonymous protein of *Babesia gibsoni* (BgTRAP), and have shown that the antisera raised against recombinant BgTRAP expressed in *Escherichia coli* inhibited the growth of parasites. In the present study, a recombinant vaccinia virus expressing the BgTRAP (VV/BgTRAP) was constructed. A specific band corresponding to a molecular mass of 80 kDa, which was similar to that of native BgTRAP on the merozoites of *B. gibsoni*, was detected in the supernatant of VV/BgTRAP-infected RK13 cells. Moreover, mice inoculated with VV/BgTRAP produced antibody that specifically reacted with the native BgTRAP on parasites. These results indicated that the recombinant vaccinia virus expressing BgTRAP might be a vaccine candidate against canine *B. gibsoni* infection.

Key words: *Babesia gibsoni*, BgTRAP, vaccine, vaccinia virus

INTRODUCTION

Babesia gibsoni is a tick-borne apicomplexan parasite that causes piroplasmosis in dogs. The disease is characterized by remittent fever, progressive anemia, hemoglobinuria, marked splenomegaly and hepatomegaly, which may lead sometimes to death. The infection has been emerged to be endemic in many regions of Asia, Africa, Europe and America (Adachi *et al.,* 1993; Casapulla *et al*., 1998; Farwell *et al*., 1982; Zhou *et al*., 2002). Despite chemotherapy is the most common for treatment of the disease, the drug resistance emerged in the endemic regions has emphasized a need for better control strategy. The development of a vaccine that would reduce or prevent the clinical symptoms of canine *B. gibsoni* infection is considered to be the best approach for controlling the disease. However, no vaccine is currently available. Therefore, there is a need to develop an effective vaccine to control *B. gibsoni* infection in dogs.

Thrombospondin-related anonymous proteins (TRAPs) are identified in several apicomplexan parasites, including *Plasmodium* spp. (Robson *et al.,* 1997; Templeton *et al.,* 1997; Trottein *et al*., 1995) *Toxoplasma gondii* (Wan *et al*., 1997) *Cryptosporidium parvum* (Spano *et al*., 1998) *Eimeria tenella* (Tomley *et al*., 1991) *Neospora caninum* (Lovett *et al*., 2000) *B. bovis* (Gaffar *et al*., 2004) and *B. gibsoni* (Zhou *et al*., 2006). Within this family, TRAPs have conserved and unique structure; typically consisting of N-terminal signal peptide, one or multiple von Willebrand factor A domain, a thrombospondin type 1 domain, a transmembrane region, and a cytoplasmic C-terminus. The protein is essential for the survival of the parasites mediating the invasion into the host cells. In our previous study, the identified BgTRAP showed bivalent cation-independent binding to canine RBCs, and the specific antiserum was found to inhibit the growth of *B. gibsoni* (Zhou *et al*., 2006). These results suggest that the BgTRAP plays a critical role in the erythrocyte invasion by *B. gibsoni* and that it might be a logical candidate for a vaccine antigen.

A live antigen delivery system has many advantages for the large-scale development of vaccines. It is

easy to produce, resistant to environmental extremes, and less expensive than other systems.　In addition, the recombinant live vaccine elicits strong host immunity against itself as well as other heterologous antigens. Recombinant vaccinia viruses have been demonstrated to be effective antigen delivery systems for various infectious diseases (Ertl and Xiang 1996; Goodman *et al.*, 2011; Moss *et al.*, 1984; Panicali *et al.*, 1983; Smith *et al.*, 1983; Tsukiyama *et al.*, 1989).　In this study, we constructed a recombinant vaccinia virus expressing BgTRAP and evaluated its immunogenicity against *B. gibsoni* in mice.

MATERIALS AND METHODS

Cells and viruses

The vaccinia virus LC16mO (mO) strain and its recombinants were propagated in rabbit kidney (RK13) cells in Eagle's minimum essential medium (Sigma, USA) supplemented with 8% fetal bovine serum (FBS).

Construction of a recombinant vaccinia virus expressing the BgTRAP or green fluorescent protein (GFP)

The recombinant vaccinia virus expressing BgTRAP (VV/BgTRAP) or GFP (VV/GFP) was constructed as early described (Nishikawa *et al.*, 2001b).　Briefly, the fragment containing the open reading frame of the *BgTRAP* gene was amplified by polymerase chain reaction (PCR) using a set of primers, 5'-ACG AAT TCA AGC ATG GCG AGG ATG AAG-3' and 5'-ACG AAT TCT CAG GCC CAC ATG GCT TCA-3' (Zhou *et al* 2006).　The PCR product was cloned into pBluescriptSK (pBS) cloning vector (Stratagene, USA) with *Eco*RI site.　The plasmid pBS/BgTRAP was digested with *Eco*RI, and the fragment (2,227 bp) containing BgTRAP was blunted using Klenow Fragment (Takara, Japan) and cloned into the *Sal*I site of the vaccinia virus transfer vector, pAK8 (Yasuda *et al.*, 1990).　On the other hand, plasmid pCX-EGFP was digested with *Eco*RI, and the fragment (732 bp) containing enhanced GFP (EGFP) was blunted using the Klenow Fragment and cloned into the *Sal*I site of pAK8. RK13 cells infected with the vaccinia virus (mO) were transfected with the recombinant transfer vectors.　Thymidine kinase-negative (TK-) viruses were isolated by a plaque assay as described previously (Yasuda *et al.*, 1990).

Indirect fluorescent antibody test (IFAT)

RK13 cells infected with VV/BgTRAP or VV/GFP were placed on slides, air-dried, and then fixed with acetone for 20 min.　The diluted (appropriate dilutions were made in 10% FBS in PBS [FBS-PBS]) anti-BgTRAP-specific rabbit serum (Zhou *et al.*, 2006) was applied as the first antibody on fixed smears and incubated for 1 hr at 37°C.　After three times washing with PBS, Alexa-Fluor 488-conjugated goat anti-rabbit immunoglobulin G (IgG) (Molecular Probes, USA) was subsequently applied (1:200 dilution in FBS-PBS) as a secondary antibody and incubated for 1 hr at 37°C.　The slides were washed three times with PBS, covered by glass slip and finally examined using a fluorescent microscope.　Furthermore, to examine the reactivity of anti-VV/BgTRAP serum with the native protein, standard immunofluorescent test was carried out as described previously (Zhou *et al.*, 2006) and imaging was made using confocal laser scanning microscope (TCS NT, Leica, Germany).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

RK13 cells infected with VV/BgTRAP or VV/GFP were cultured on 6-well plates with a 0.5 ml medium. The culture medium was harvested by centrifugation, and the cells were suspended in 1 ml PBS.　The cells were washed three times and lysed by sonication.　The cell lysates or culture supernatants were mixed with an equal volume of a 2×SDS gel-loading buffer under reducing conditions.　The samples were boiled for 5 min, and each 10 µl of sample was then subjected to SDS-PAGE.　After SDS-PAGE, the protein bands in the gel were electrically transferred to a membrane (Immobilon transfer membrane, Millipore, USA) .　The membrane was blocked with PBS containing 3% skimmed milk and then incubated with anti-BgTRAP rabbit serum (Zhou *et al.*, 2006) diluted 1:200 with PBS containing 3% skimmed milk at 37°C for 1 hr.　The membrane was washed three times and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG diluted 1:2,000 with PBS containing 3% skimmed milk at 37°C for 1 hr.　After washing

three times with PBS, the bands recognized by a specific antibody were visualized by incubation with 0.5 mg/ml 3',3-diaminobenzine in PBS containing 0.03% H_2O_2.

Vaccination

Female BALB/c mice (6 weeks old, Clea Japan) were inoculated intraperitoneally (i.p.) with either VV/BgTRAP or VV/GFP at dose of 1×10^6 plaque-forming units (pfu) per mouse. Mice were boosted with the same inoculum 14 days after the first inoculation. Serum was collected at 1 week intervals from each mouse. All animals experiments described in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals Promulgated by Obihiro University of Agriculture and Veterinary Medicine.

Measurement of *B. gibsoni*-specific antibody

The BgTRAP-specific immunoglobulin response in immunized mice was measured by the enzyme-linked immunosorbent assay (ELISA). Briefly, 2 µg/ml purified GST-BgTRAP or control GST (Zhou *et al.*, 2006) diluted in a 50 mM carbonate-bicarbonate buffer (pH 9.6) to, were coated on 96-well ELISA plate (Nunc, Denmark) and incubated at 4˚C overnight. The plates were washed once with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 3% skimmed milk for 1 hr at 37˚C. After washing once with PBS-T, 50 µl of serum samples diluted to 1:100 in PBS containing 3% skimmed milk was added to duplicate wells for each sample and incubated at 37˚C for 1 hr. After washing 6 times with PBS-T, the plate was incubated with HRP-conjugated goat anti-mouse IgG antibody at 37˚C for 1 hr. The plates were washed 6 times with PBS-T, 100 µl of substrate was added to each well and incubated at room temperature for 1 hr. The absorbance at 415 nm was measured by using an ELISA plate reader (Corona, Ibaraki, Japan) and is shown as the distance between the GST-BgTRAP and control GST. Then, the plates were washed 6 times with PBS-T, and 100 µl of a substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.3 mg/ml of 2,2'-azide-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma), and 0.01% of 30% H_2O_2] was added to each well. The absorbance at 415 nm was measured after 1 hr of incubation at room temperature by using MTP-500 microplate reader (Corona Electric, Tokyo, Japan).

RESULTS

In order to develop an effective recombinant vaccine against *B. gibsoni* infection, a recombinant vaccinia virus expressing BgTRAP (VV/BgTRAP) was constructed. The *BgTRAP* gene was inserted into the *TK* gene of the vaccinia virus mO strain under the control of the early-late promoter for the vaccinia virus 7.5 kDa polypeptide. To determine whether the BgTRAP was expressed in RK13 cells by the recombinant virus, the VV/BgTRAP-infected cells were examined by IFAT using anti-BgTRAP mouse sera.
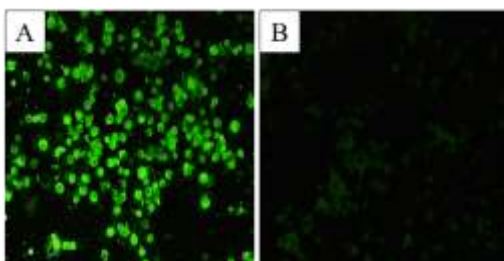

Fig. 1. IFAT of recombinant BgTRAP expressed in RK13 cells by a vaccinia virus. (A) VV/BgTRAP-infected cells or (B) VV/wt were allowed to infect RK13 cells, and the test was performed 48 h post-infection with anti-BgTRAP-specific rabbit serum followed by Alexa Flour-488-conjugated secondary antibodies.
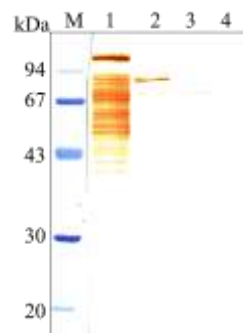

Fig. 2. Western blot analysis of a recombinant vaccinia virus with anti-BgTRAP rabbit serum. Cell lysates of RK13 cells infected with VV/BgTRAP (lane 1), culture supernatants of RK13 cells infected with VV/BgTRAP (lane 2), cell lysates of RK13 cells infected with VV/GFP (lane 3), and culture supernatants of RK13 cells infected with VV/GFP (lane 4) were separated by SDS-polyacrylamide gel electro-phoresis following Western blot analysis using anti-BgTRAP rabbit serum as the primary antibody. Lane M, molecular mass markers.

As shown in Fig 1, specific fluorescence was observed in VV/BgTRAP-infected cells but not in VV/WT-infected cells. Next, the molecular mass of the BgTRAP expressed by recombinant VV/BgTRAP was determined by Western blot analysis. A specific band with a molecular mass of 80 kDa was detected in the supernatants of VV/BgTRAP-infected RK13 cells (Fig. 2), which was similar to that of native BgTRAP on the merozoites of *B. gibsoni* (Zhou *et al.*, 2006). These results indicated the success in expressing BgTRAP using vaccinia virus system.
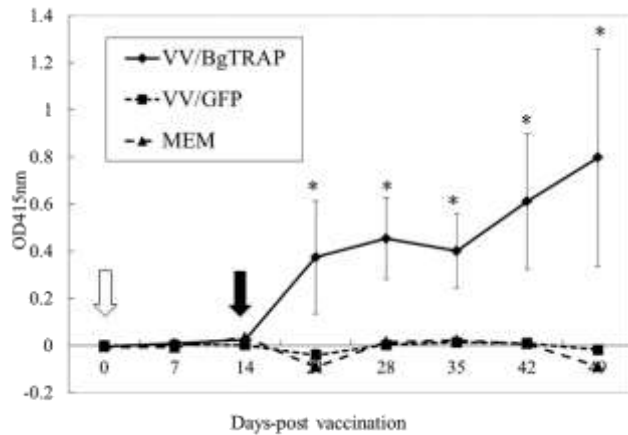

Fig. 3. *B. gibsoni*-specific antibody responses of mice vaccinated with recombinant vaccinia viruses and control mice inoculated with VV/GFP. Mice were inoculated with 1×10^6 pfu of vaccinia viruses on days 0 and 13. Serum samples were collected at 1 week intervals from each mouse, and *B. gibsoni*-specific antibody responses were measured by ELISA. White arrow indicates vaccination and black arrow indicates boost. *; $P < 0.05$.
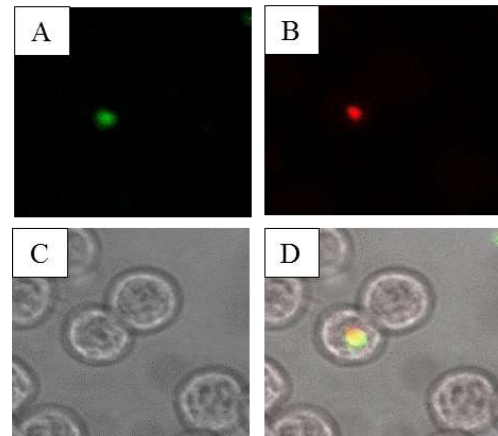

Fig. 4. Confocal laser microscopic observation of parasites with mouse anti-VV/BgTRAP serum. (A) Immunofluorescent staining of *B. gibsoni* merozoites with mouse anti-VV/BgTRAP serum. (B) Propidium iodide staining of *B. gibsoni* merozoite nuclei. (C) The phase-contrast images of *B. gibsoni* merozoites. (D) Panels (A) and (B) were overlaid on panel (C).

Furthermore, mice inoculated with recombinant VV/BgTRAP but not in mice inoculated with the control virus VV/GFP showed specific immune antibody to BgTRAP as determined by ELISA (Fig. 3). Notably, the antibody response against BgTRAP was gradually increased after boosting with recombinant VV/BgTRAP. Next, the same antisera were used to identify the native protein of BgTRAP by IFAT. Confocal laser microscopic observation demonstrated cytoplasmic fluorescent staining corresponding to BgTRAP localization in the parasites (Fig. 4).

DISCUSSION

Vaccinia viruses have been widely used as live vectors to express foreign genes of various infectious pathogens. In general, the immunization of laboratory animals or natural host animals with these recombinant vaccinia viruses could induce neutralizing antibodies and protect the animals from challenge infections with corresponding infectious viruses. Recently, vaccinia virus vector has been also used as live vector to express foreign genes from protozoan parasites, and demonstrated that the animals inoculated recombinant vaccinia vaccines could induce protective immunity against virulent parasite infections (Honda *et al.*, 1998; Miyahara *et al.*, 1998; Nishikawa *et al.*, 2001a).

The TRAP-homologue was identified in all apicomplexan parasites as essential molecule for their survival mediating the invasion into the host cells. In *Plasmodium*, the deletion of TRAP was not possible in due to its crucial role in the sporozoite motility and liver cell invasion (Naitza *et al.*, 1998) as well as in the merozoite invasion into host erythrocytes. However, a recent study has shown that the molecular motor of cell invasion and gliding motility across apicomplexan parasites are conserved (Baum *et al.*, 2006). Previously, we identified the TRAP-homologue from *B. gibsoni* and provided direct evidence of this protein is capable of binding canine erythrocytes (Zhou *et al.*, 2006). Interestingly, specific anti-BgTRAP serum

has shown significant inhibition of the growth of *B. gibsoni* in the infected severe combined immune deficiency mice given canine erythrocyte (Zhou *et al*., 2006).

In this study, we constructed a recombinant vaccinia virus expressing BgTRAP and examined its immunogenicity in mice.   A specific band of 80 kDa was detected in the supernatants of VV/BgTRAP-infected RK13 cells.   The extra bands from the cell lysate of RK13 cells infected with VV/BgTRAP suggest that it undergoes limited processing and that only mature BgTRAP was secreted into the supernatant.   The specific antibody response against the BgTRAP was gradually increased after boosting with VV/BgTRAP.   These sera detected the native BgTRAP by IFAT and the confocal microscopic observation revealed the cytoplasmic localization of the protein near the apical end.   These were consistent with our previous findings demonstrating that BgTRAP has apparent 80 kDa protein size in Western blotting and is localized at the apical end of the merozoite (Zhou *et al*., 2006).   These results indicated that vaccinia virus expressing BgTRAP could induce specific immune response to the corresponding protein in mice. Our further study is to examine the potential inhibitory effects of antibody produced in mice by VV/BgTRAP against *B. gibsoni* parasites.

The mechanisms of protective immunity to babesial parasites are hypothesized to require both innate and adaptive responses.   The CD4$^+$ T cells play the central role in protection secreting required cytokines for macrophages and specific antibody production.   The clearance of infected erythrocytes occurs by activated splenic macrophages (Brown 2001; Brown and Palmer 1999; Hemmer *et al*., 2000) and by neutralizing antibodies directed against extracellular merozoites.   Cellular immunity seemed to be very important in the control of intracellular parasites due to the fact that the parasites are hidden within the erythrocytes and are not accessible to humoral response.   Therefore, the ideal *Babesia* vaccine should contain components that activate cellular and humoral responses (Brown *et al*., 2006).   We believe that the capability of anti-BgTRAP serum in disrupting the invasion of parasites into their host cells coupled with the efficacy of vaccine virus vector in triggering strong cellular response (Reyes-Sandoval *et al*., 2007) can be promising for development of molecular vaccine against *B. gibsoni* infection.

In conclusion, the recombinant vaccinia virus expressing BgTRAP was shown to be immunogenic in mice inducing specific response to BgTRAP.   Our data indicated that VV/BgTRAP might be a vaccine candidate against canine *B. gibsoni* infection.   Evaluation the potency of VV/BgTRAP as a live vaccine for the control of canine babesiosis caused by *B. gibsoni* is desired.

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