Construction of recombinant vaccinia virus targeting *Babesia gibsoni* infection and evaluation of its immunogenicity

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犬バベシア感染症を標的とした組換えワクシニアウイルスベクターの作製とその免疫原性の評価

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Abbreviations

ABTS: 2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonic acid

BgTRAP: *Babesia gibsoni* thrombospondin-related adhesive protein

BSA: bovine serum albumin

bp: base pair

BUdR: 5-bromo-2'-deoxyuridine

cDNA: complementary DNA

DNA: deoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

EMEM: Eagle's minimum essential medium

FBS: fetal bovine serum

GFP: green fluorescent protein

GST: glutathione *S*-transferase

HRPO: horse radish peroxidase

IFAT: indirect fluorescent antibody test

Ig: immunoglobulin

kDa: kilo Dalton

mRNA: messenger RNA

NRS: normal rabbit serum

OD: optical density

ORF: open reading frame

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFU: plaque forming unit

PI: propidium iodide

SCID: severe combined immunodeficiency

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TBS: tris-buffered saline

TK: thymidine kinase

TRAP: thrombospondin related adhesive protein

VV: vaccinia virus

wt: wild type

Abstract

Vaccinia virus, no longer required for immunization against smallpox, now serves as a unique vector for expressing foreign genes within the cytoplasm of mammalian cells. The vaccine potential of recombinant vaccinia virus has been realized in the form of an effective oral wild-life rabies vaccine. A genetically altered vaccinia virus that is unable to replicate in mammalian cells and produces diminished cytopathic effects retains the capacity for high-level gene expression and immunogenicity while promising exceptional safety for laboratory workers and potential vaccine recipients. On the other hand, *Babesia gibsoni* is a tick-borne apicomplexan parasite that causes piroplasmosis in dogs. No vaccine against *B. gibsoni* infection is currently available. Therefore, there is a need to develop an effective vaccine to control *B. gibsoni* infection in dogs. Previously, *Babesia gibsoni* thrombospondin related adhesive protein (BgTRAP) is expected to be important for erythrocyte invasion by *B. gibsoni* merozoite. In this research, a recombinant vaccinia virus expressing BgTRAP (VV/BgTRAP) was constructed. A specific band with molecular mass of about 80 kDa, which is similar to that of native BgTRAP on merozoites of *B. gibsoni*, was detected in the supernatants of VV/BgTRAP-infected RK13 cells. The mice inoculated with VV/BgTRAP produced a specific anti-BgTRAP response. The antiserum against recombinant VV/BgTRAP 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.

Introduction

Babesia gibsoni is a tick-borne apicomplexan parasite that causes piroplasmosis in dogs. The disease is characterized by remittent fever, progressive anemia, hemoglobinuria, and marked splenomegaly and hepatomegaly; in addition, sometimes, it causes death (Casapulla et al., 1998; Wozaniak et al., 1997; Yamane et al., Yamane et al.,1993). *B. gibsoni* infection is endemic in many regions of Asia, Africa, Europe, and America (Irizarry-Rovira et al., 2001; Macintire et al., 2002; Zhou et al., 2002). Recently, this disease has frequently been observed in companion animals, becoming a significant problem from a clinical point of view (Adachi et al., 1993; Farwell et al., 1982).

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. For intracellular protozoan infections such as malaria or babesiosis, it is known that the induction of immune responses at both the humoral and cellular levels is required for protection against these pathogens (Brown et al., 1999; Tsuji et al., 2003). Therefore, a vaccine may be needed to induce both types of immune responses and provide optimal protection.

The traditional approach for vaccine development is based on recombinant proteins. However, a recombinant protein-based vaccine is poorly immunogenic. As such, many recombinant protein-based vaccines require the addition of an adjuvant to boost immune responses. Most of the experimental protozoan vaccines are currently using adjuvants that have yet to be used in licensed vaccines. In addition, many antigenic targets are highly structured and proper conformation

seems to be required to generate a functional immune response. This leads to challenges in developing the purification process where refolding and isolation of proper conformers often results in complex manufacturing processes and low yields. Another challenge is the long-term stability of recombinant proteins when they are formulated in adjuvants at ambient temperatures. Viral vectors, on the other hand, appear to be capable of inducing both antibody and T-cell-mediated immunity in the absence of an adjuvant (Zarling et al., 1986). Furthermore, complex process development may not be required for viral-vectored vaccines, which usually have a consistent purification process, irrespective of the transgene they express (Earl et al., 1986; Lathe et al., 1987). As these vaccines use the eukaryotic cellular machinery to generate the antigenic targets, it may be possible to generate antigens with native conformation. Finally, some of the viral vectors have the capacity to deliver more than one gene. Thus, a single viral vectored construct may contain multiple antigens from the different parasite life stages and would have the potential to induce a broad protective immunity. Significant manufacturing cost savings could also be realized (Li et al., 2007).

Vaccinia virus was selected as a vector because the large genome size and absence of stringent packaging constraints allow the introduction of foreign DNA without loss of infectivity (Panocali et al., 1982; Mackett et al., 1982; Smith et al., 1983). In addition, vaccinia virus has a wide host range, permitting veterinary and medical applications, and has been used successfully worldwide for mass immunization against smallpox (Fenner et al., 1988; Wittek, R., 1980). In addition, recombinant vaccinia virus has been demonstrated to be an effective antigen delivery system for viral infectious diseases in many species (Brochier et al.,

1991; Ertl et al., 1996; Moss et al., 1984; Panicali et al., 1983; Smith et al., 1983; Tsukiyama et al., 1989) and for parasite infections (Honda et al., 1998; Schneider et al., 1998).

The strategy of insertion and expressing foreign DNA in vaccinia virus has been described in detail (Moss et al., 1983). Essentially, the construction of recombinant vaccinia virus is carried out in two stages. First, recombinant DNA techniques are used to construct a transfer plasmid containing a chimeric gene flanked by vaccinia virus DNA. The chimeric gene has the transcriptional regulatory signals and RNA start site of a vaccinia virus gene (Venkatesan et al., 1981; Venkatesan et al., 1982; Weir et al., 1983) adjacent to the translational start site and foreign protein coding sequence of a foreign gene. The next stage is the insertion of the chimeric gene into vaccinia virus. This occurs by homologous recombination in cells that have been infected with vaccinia virus and transfected with the plasmid containing the chimeric gene (Sam et al., 1981; Nakano et al., 1982; Weir et al., 1982). Any nonessential region of the vaccinia genome could be used to flank the chimeric gene. The vaccinia virus gene for thymidine kinase (TK) (Weir et al., 1983) was one of useful site for homologous recombination, because recombinants will then have a TK-negative phenotype and can be selected by plaque formation in the presence of 5-bromo-2'-deoxyuridine (BUdR). This process has been simplified by the construction of plasmid insertion vectors that have properly positioned restriction.

Some of the protozoan antigens are involved in protective immunity of host.

Therefore, antigen genes, which could induce the host protective immunity, have been searched by performing genomic analyses of protozoa. Thrombospondin related adhesive proteins (TRAPs) are a conserved family identified in several apicomplexans, including *Plasmodium*, *Toxoplasma gondii*, *Cryptosporidium parvum*, *Eimeria tenella*, *Neospora caninum*, and *Babesia bovis* (Gaffar et al., 2004; Lovett et al., 2000; Robson et al., 1997; Spano et al., 1998; Templeton et al., 1997; Tomeley et al., 1991; Trottein et al., 1995; Wan et al., 1997). Previously, my laboratory has identified and characterized a *B. gibsoni* TRAP (BgTRAP) (Zhou et al., 2006). The amino acid sequence of BgTRAP consists of several typical regions, including a signal peptide, a vonWillebrand factor A domain, a thrombospondin type 1 domain, a transmembrane region, and a cytoplasmic C-terminus. The *B. gibsoni*-infected dog serum recognized recombinant BgTRAP expressed in *Escherichia coli* by Western blotting. The antiserum against recombinant BgTRAP recognized an 80 kDa protein in the lysate of infected erythrocytes (RBCs), which was detectable in the micronemal area of the parasites by confocal microscopic observation. The BgTRAP bound to canine RBC, and the specific antiserum was found to inhibit the growth of *B. gibsoni* in the infected severe combined immune deficiency mice given canine RBC. These results suggest that the BgTRAP is a new member of TRAP family identified from the merozoites of *B. gibsoni* and functionally important in merozoite invasion; therefore, this protein may be useful as a vaccine candidate against canine *B. gibsoni* infection (Zhou et al., 2006).

In this study, I constructed recombinant vaccinia virus expressing BgTRAP and evaluated its immunogenicity against *B. gibsoni* infection.

Materials and methods

1. Viruses and cell culture

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

2. Parasites and cell culture

B. gibsoni isolated from a hunting dog of Hyogo Prefecture, Japan, designated as NRCPD strain, was maintained in splenectomized beagles as described earlier (Fukumoto et al., 2001; Ishimine et al., 1978). *B. gibsoni*-infected dog erythrocytes were collected from the experimentally infected dogs at peak parasitemia (14%) and stored at −80°C.

3. Cloning of BgTRAP gene

The fragment containing the open reading frame (ORF) of the BgTRAP gene was amplified by polymerase chain reaction (PCR) using a set of primers with introduced *Eco*RI sites, 5'-ACGAATTCAAGCATGGCGAGGATGAAG-3' and 5'-ACGAATTC TCAGGCCCACATGGCTTCA-3'. The PCR product was cloned into *Eco*RI site of the cloning vector, pBluescript SK (pBS) (Stratagene, USA). The resulting plasmid was designated as pBS/BgTRAP.

4. Construction of recombinant vaccinia virus expressing BgTRAP or green fluorescent protein (GFP)

The recombinant vaccinia virus, which express BgTRAP (VV/BgTRAP) or GFP (VV/GFP), was constructed as follows. The plasmid pBS/BgTRAP was cut 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 vaccnia virus transfer vector, pAK8 (Yasuda et al., 1990). On the other hand, plasmid pCX-EGFP was cut with *Eco*RI, and the fragment (732 bp) containing EGFP was blunted using the Klenow Fragment and cloned into the *Sal*I site of pAK8. The structure of recombinant plasmid pAK8/BgTRAP or pAK8/GFP was checked by restriction enzyme analysis. RK13 cells infected with vaccinia virus LC16mO (Sugimoto et al, 1985) strain were transfected with the pAK8/BgTRAP or pAK8/GFP using lipofectine reagent. Thymidine kinase negative (TK-) viruses were isolated by plaque assay on 143TK- cells in the presence of BUdR at concentration of 100 μg/ml (Yasuda et al., 1990). The recombinant vaccinia virus expressing BgTRAP (VV/BgTRAP) or GFP (VV/GFP) was propagated in RK13 cells in EMEM supplemented with 8% FBS. To analyze the expression of BgTRAP in vitro, RK13 cells were inoculated with VV/BgTRAP or VV/GFP per cell. Two days after inoculation, the cells were harvested and then subjected to indirect fluorescent antibody test (IFAT) or Western blotting as described above.

5. Expression and purification of the recombinant BgTRAP (rBgTRAP) in *E. coli*

The ORF of the BgTRAP gene in the pBluescriptSK(+) vector was subcloned into an *E. coli* expression vector, pGEX-4T-3 (Amersham Pharmacia Biotech, UK). The resulting plasmid was checked for accurate insertion by sequencing and designated as

the pGEX-4T-3/BgTRAP. The BgTRAP gene was expressed as a glutathione *S*-transferase (GST)-fusion protein in the *E. coli* BL21 (DE3) strain according to the manufacturer's instructions (Amersham Pharmacia Biotech, UK). The resulting *E. coli* cells were washed three times with phosphate-buffered saline (PBS), lysed in 1% Triton X-100–PBS, sonicated, and then centrifuged at 10,000 rpm for 10 min at 4°C. The soluble GST fusion protein was purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions.

6. Production of anti-rBgTRAP serum

Five hundred microgram of the rBgTRAP or rGST in Freund's complete adjuvant (Difco Laboratories, USA) was subcutaneously injected into a rabbit (Japanese white rabbit, 2.5 kg). The same antigen in Freund's incomplete adjuvant (Difco Laboratories, USA) was subcutaneously injected into the rabbit on day 14 and again on day 28. The production of rabbit antibodies against rBgTRAP was periodically estimated using ELISA. Serum from immunized rabbit was collected 14 days after the last immunization.

7. Detection of BgTRAP expressed in VV/BgTRAP-infected cells by 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 the fixed smears and incubated for 1 hr at 37°C. After three washings with PBS, Alexa-Fluor® 488-conjugated goat anti-rabbit

immunoglobin G (IgG) (Molecular Probes, USA) was subsequently applied (1:200 dilution in FBS–PBS) as a secondary antibody and incubated for another 1 hr at 37°C. After three washings with PBS, the glass slides were covered with a glass cover-slip, and then examined under a fluorescent microscope.

8. Preparation of samples for sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE).

RK13 cells infected with VV/BgTRAP or VV/GFP were cultured on 6-well plates with 0.5 ml EMEM. At the end of culture, the culture medium was harvested and centrifuge at 5,000 rpm for 10 min at 4°C. The culture supernatants were collected and the cells were harvested in 1 ml PBS. Then, the cells were washed three times with PBS and resuspended with 100 μl of cold PBS containing 0.1% Triton X-100. After sonication, the samples were kept at room temperature for 30 min, and then repeatedly frozen and thawed three times. After centrifuging at 15,000 rpm for 30 min at 4°C, the supernatants were collected. The lysate of *B. gibsoni*-infected dog RBCs were used to identify the molecular weight of native BgTRAP in merozoites of *B. gibsoni*. The lysates of *B. gibsoni*-infected dog RBCs were treated with 0.83% NH_4Cl solution for 10 min at 37°C and then washed three times with cold PBS (Martin et al., 1971). The pellets were suspended in PBS.

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

Samples were resuspended in equal volume of SDS gel-loading buffer [50 mM Tris-HCl (pH6.8), 50 mM 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10%

glycerol] under reducing conditions. The samples were boiled for 5 min, and each 10 μl of sample was then analyzed by 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 TBS-T containing 3% skim milk and then incubated with anti-rBgTRAP rabbit serum diluted 1:200 with TBS-T containing 3% skim milk at 37°C for 1 hr. The membrane was washed three times with TBS-T for 30 min and then incubated with HRPO-conjugated goat anti-rabbit IgG diluted 1:2,000 with TBST containing 3% skim milk at 37°C for 1 hr. After washing tree times with TBS-T for 30 min, the bands recognized by the specific antibody were visualized by incubation with 0.5 mg/ml 3',3-diaminobenzine in PBS containing 0.03% H_2O_2.

10. Preparation of VV/BgTRAP culture supernatants antigen for ELISA

RK13 cells infected with VV/BgTRAP or VV/GFP were cultured on 6-well plates with 0.5 ml EMEM without FBS. At the end of culture, the culture medium was harvested and centrifuged at 5,000 rpm for 10 min at 4°C. The culture supernatants were collected and ultracentrifuged at 35,000 rpm for 2 hrs. After ultracentrifugation, the supernatants were added to 6-well plates and irradiated with ultraviolet light for 1 hr.

11. Serum samples used for ELISA

Dog serum samples used in this study were as follows: 25 sera from specific pathogen-free (SPF) dogs (Nihonnosan, Japan); sequential serum samples (0–541 days post-infection) from a dog experimentally infected with *B. gibsoni* NRCPD strain; 2 sera each from dogs experimentally infected with *B. canis canis*, *B. canis vogeli*, and *B. canis rossi*; 2 sera from dogs experimentally infected with *Leishmania infantum*; 2 sera

from dogs infected with *Neospora caninum.*

12. Detection of antibody to BgTRAP in dogs infected with *B. gibsoni* and other closely related protozoan parasites by ELISA using vaccinia virus-expressed antigen

Individual wells of a microtiter plate were coated with BgTRAP or control GFP expressed by vaccinia virus as antigen for ELISA. The antigen was diluted in 1:20 in 50 mM carbonate-bicarbonate buffer (pH9.6), and 50 μl aliquots of the diluted antigen were added to each well of a 96-well ELISA plate (Nunc, Denmark). The plate was incubated at 4°C overnight and washed once with PBS containing 0.05% Tween 20 (PBS-T). Then, residual binding sites were blocked with PBS containing 3% skim milk for 1 hr at 37°C. Each well was washed once with PBS-T, 50 μl of serum samples diluted 1:100 with PBS containing 3% skim milk were added to duplicate wells for each sample. The plate was incubated at 37°C for 1 hr. After washing 6 times with PBS-T, the plate was incubated with HRPO-conjugated goat anti-dog IgG antibody (Bethy Laboratories, USA) diluted 1:4,000 with PBS containing 3% skim milk at 37°C for 1 hr. After washing 6 times with PBS-T, 100 μl of substrate [52 mM citric acid, 103nM Na_2HPO_4, 0.003% H_2O_2, 0.05 mg of 2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma-Aldrich, USA) per ml] was added to each well, and incubated at room temperature for 1 hr. The absorbance was measured at 415 nm by using ELISA plate reader (Corona, Japan) and shown as the distance between the BgTRAP and control GFP.

13. Immunization of mice with VV/BgTRAP

For preparation of viral inoculum, recombinant vaccinia virus that express VV/BgTRAP was propagated in RK13 cells in OPTI-MEM®I (Invitrigen, Gibco, USA) without FBS. Female BALB/c mice (6-week-old) were purchased from a commercial supplier (Clea Japan). One group of mice was inoculated intraperitoneally (i.p.) with VV/BgTRAP. Another group of mice inoculated i.p. with VV/GFP was used as viral controls. Doses of vaccinia viruses were 1×10^6 plaque forming units (pfu). Mice were boosted with the same inoculum 14 days after the first inoculation. Serum was collected at 1 week intervals from each mouse.

14. Determination of antibody response by ELISA

BgTRAP specific immunoglobulin level in mouse serum was measured by ELISA. Purified GST-BgTRAP or control GST expressed by *E. coli* was diluted in 50 mM carbonate-bicarbonate buffer (pH9.6) to 2 μg/ml, and 50 μl aliquots of the diluted antigen were added to each well of a 96-well ELISA plate (Nunc, Denmark). The plate was incubated at 4°C overnight and washed once with PBS containing 0.05% Tween 20 (PBS-T). Then, residual binding sites were blocked with PBS containing 3% skim milk for 1 hr at 37°C. Each well was washed once with PBS-T, 50 μl of serum samples diluted 1:100 with PBS containing 3% skim milk were added to duplicate wells for each sample. The plate was incubated at 37°C for 1 hr. After washing 6 times with PBS-T, the plate was incubated with HRPO-conjugated goat anti-mouse IgG, IgG1 and IgG2a antibodies diluted 1:4,000 with PBS containing 3% skim milk at 37°C for 1 hr. After washing 6 times with PBS-T, 100 μl of substrate [52 mM citric acid, 103 nM Na_2HPO_4, 0.003% H_2O_2, 0.05 mg of 2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonic acid (ABTS,

Sigma-Aldrich, USA) per ml] was added to each well, and incubated at room temperature for 1 hr. The absorbance at 415 nm was measured using ELISA plate reader (Corona, Japan) and shown as the distance between the GST-BgTRAP and control GST.

15. IFAT and confocal laser microscopic observation

The IFAT was carried out to identify the BgTRAP on *B. gibsoni* merozoites using anti-VV/BgTRAP antibody. Thin blood smear films of *B. gibsoni*-infected blood samples collected from a *B. gibsoni*-infected dog were fixed with methanol containing 2.5% acetone for 20 min. The diluted (appropriate dilutions were made in 10% fetal calf serum in PBS (FCS–PBS)) anti-VV/BgTRAP-specific mouse serum was applied as the first antibody on the fixed smears and incubated for 30 min at 37°C. After three washings with PBS, Alexa-Fluor® 488-conjugated goat anti-mouse IgG (Molecular Probes, USA) was subsequently applied (1:1,000 dilution in FBS–PBS) as a secondary antibody and incubated for another 30 min at 37°C. The slides were washed three times with PBS and incubated with 6.25 μg/ml propidium iodide (PI) (Molecular Probes, USA) containing 50 μg/ml RNase A (Qiagen, Germany) for 10 min at 37°C. After two washings with PBS, the glass slides were mounted by adding 200 μl of a 50% glycerol–PBS (v/v) solution and covering with a glass cover-slip. The slides were examined under a confocal laser scanning microscope (Leica microsystems, Germany).

16. Statistical analyses

Levels of significance of the difference between groups were determined by the student's t-*t*est. $P<0.05$ was considered statistically significant.

All animal 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.

Results

1. Construction of recombinant vaccinia virus expressing BgTRAP

In order to development an effective recombinant vaccine against *B. gibsoni* infection in dogs, a recombinant vaccinia virus expressing BgTRAP was constructed. The BgTRAP gene was inserted into the TK gene of 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 recombinant vaccinia virus, the VV/BgTRAP-infected cells were examined by IFAT using anti-rBgTRAP mouse sera. As shown in Fig. 1, specific fluorescence was observed in VV/BgTRAP-infected cells, but not in VV/WT-infected cells.

To determine the molecular mass of BgTRAP expressed by recombinant VV/BgTRAP, Western blot analysis was performed. A specific band with molecular mass of 80 kDa, which was similar to that of native BgTRAP on merozoites of *B. gibsoni*, was detected in the supernatants of VV/BgTRAP-infected RK13 cells (Fig. 2).

2. Evaluation of the antigenicity of BgTRAP expressed by recombinant vaccinia virus

To evaluate the antigenicity of BgTRAP expressed by recombinant vaccinia virus, the BgTRAP was tested in ELISA using sera from dogs experimentally infected with *B. gibsoni* and other closely related protozoan parasites. As shown in Fig. 4, 10 sera from dogs infected with *B. gibsoni* were positive (lane 1, optical density > 0.05). In contrast the following sera were negative (OD<0.05); 2 sera from dogs infected with *B. canis vogeli* (lane 2), 3 sera from dogs infected with *B. canis rossi* (lane 3), 3 sera from dogs

infected with *B. canis canis* (lane 4), 3 sera from dogs infected with *L. infantum* (lane 5), 3 sera from dogs infected with *N. caninum* (lane 6), or 3 sera from dogs infected with *L. infantum*, and 25 sera from SPF dogs (lane 7). As shown in Fig. 3, a dog experimentally infected with *B. gibsoni* gradually developed antibody response to BgTRAP. The high antibody titer was maintained until 541 days post-infection even when the dog became chronically infected with low level of parasitemia.

3. Immune responses to BgTRAP in mice inoculated with recombinant vaccnia virus

To confirm the immunogenicity of BgTRAP expressed in recombinant vaccinia virus, one group of mice was inoculated intraperitoneally (i.p.) with VV/BgTRAP, and another group of mice inoculated i.p. with VV/GFP was used as viral controls. Serum samples collected from these mice were analyzed using ELISA. Fig. 5a shows the specific antibody responses determined by ELISA with GST-BgTRAP expressed in *E. coli*. The IgG response against BgTRAP was gradually increased after boosting inoculation with recombinant VV/BgTRAP. Control group inoculated with VV/GFP did not develop specific antibody response against BgTRAP. The IgG subclasses were considered to be a representative marker for the Th1/Th2 type immune responses (Briere et al., 1994; Kawano et al., 1994; Stevens et al., 1988). There was an increase in both IgG1 and IgG2a in mice immunized with VV/BgTRAP, with IgG2a having a greater increase than IgG1 (Fig. 5b, 5c). On the other hand, the sera collected from mice inoculated with VV/BgTRAP were examined by IFAT using *B. gibsoni* merozoites as an antigen. The BgTRAP-specific antibody induced in mice reacted strongly with intact *B. gibsoni* merozoites, as judged by IFAT (Fig. 6). Specific fluorescence appears to be distributed on the micronemal area of the parasites.

Discussion

Vaccinia virus has been widely used as a live vector to express foreign genes mainly from other infectious viruses. In general, immunization of laboratory animals or natural host animals with these recombinant vaccnia viruses could induce neutralizing antibodies and could 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; Miyahira et al., 1998; Nishikawa et al., 2001).

TRAP from *Plasmodium* is the essential adhesion needed for sporozoite motility and for liver cell invasion (Naitza et al., 1998). Moreover, recent findings including the identification of *Plasmodium* merozoite TRAP-homologue have shown a conserved molecular motor of cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites (Baum et al., 2006). Previously, Zhou et al. (2006) identified TRAP-homologue from *B. gibsoni* and provided a direct evidence of this protein binding to erythrocytes. The BgTRAP showed a bivalent cation-independent binding to canine RBC, and the specific antiserum was found to inhibit the growth of *B. gibsoni* in the infected sever combined immune deficiency mice given canine RBC (Zhou et al., 2006). These results indicated that BgTRAP might be useful vaccine candidate controlling canine *B. gibsoni* infection.

In this study, I constructed recombinant vaccinia virus expressing BgTRAP. In Western blotting, a specific band of 80 kDa, which was identical to that of native BgTRAP expressed in *B. gibsoni*, was detected in the supernatants of VV/BgTRAP infected RK13 cells. The extra bands from cell lysate of RK13 cells infected with

VV/BgTRAP suggest that it undergoes limited processing and only mature BgTRAP was secreted into supernatant. To evaluate whether the BgTRAP expressed in recombinant vaccinia virus can be a suitable antigen of *B. gibsoni* infection in dogs, the BgTRAP was tested in ELISA using sera from dogs experimentally infected with *B. gibsoni* and other closely related protozoan parasites. The ELISA with BgTRAP showed the low level of background and could differentiate *B. gibsoni*-infected dogs from normal dogs or other related protozoan parasites-infected dogs. In the sequential serum samples derived from a dog experimentally infected with *B. gibsoni*, ELISA antibody titers become positive at day 8 post infection and gradually increased consistent with progression of *B. gibsoni* infection. These results indicated that the BgTRAP expressed in recombinant vaccinia virus could be used as an effective antigen for *B. gibsoni* infection in dogs.

To examine the immunogenicity of VV/BgTRAP, mice (n=4) were inoculated i.p. with VV/BgTRAP. IgG antibody response against BgTRAP gradually increased after boosting immunization with VV/BgTRAP. These antibody responses were significantly higher than control mice group. These results demonstrated that BgTRAP expressed in mice by vaccinia virus vector is similar to the native BgTRAP in terms of its molecular structure and antigenicity. I also determined the type of immune response elicited in mice by the measurement of the ratio of IgG1 and IgG2a antibodies to BgTRAP. The IgG subclasses were considered to be a representive marker for the Th1/Th2 type immune responses (Briere et al., 1994; Kawano et al., 1994; Stevens et al., 1988). The mice immunized with VV/BgTRAP showed an increase of IgG2a over IgG1 subclass. These results indicated an induction of Th1 type T-cell-mediated immune response in mice immunized with VV/BgTRAP.

Serum samples collected from mice inoculated with VV/BgTRAP were examined by IFAT using *B. gibsoni* merozoites as an antigen. Confocal microscopic analysis

suggested that the antiserum against the recombinant BgTRAP was detectable in the micronemal area of the parasite. These results demonstrated that serum samples collected from mice inoculated with VV/BgTRAP recognized *B. gibsoni* and VV/BgTRAP could be useful candidate for development of a vaccine for control of canine *B. gibsoni* infection.

The mechanisms of immunity to babesial parasites are hypothesized to require both innate and adaptive responses that include both neutralizing antibody and $CD4^+$ T cells. Because *Babesia* parasites only infect erythrocytes, the adaptive immune response to subsequent infection and protection against clinical disease is dependent on presentation of parasite antigens by antigen presenting cells to $CD4^+$ T lymphocytes (Brown and Palmer, 1999; Homer et al., 2000; Brown, 2001). Control of *Babesia* infection is likely to be mediated by neutralizing antibodies directed against extracellular merozoites and by destruction of infected erythrocytes by activated splenic macrophages (Brown and Palmer, 1999; Brown, 2001). Because BgTRAP is expected to be important for erythrocyte invasion by *B. gibsoni* merozoite (Zhou et al., 2006), inducing antibodies to BgTRAP by VV/BgTRAP could be useful for host immune system.

In conclusion, a recombinant vaccinia virus expressing BgTRAP was constructed and its antigenicity in a laboratory animal was evaluated. These results indicated that the recombinant vaccinia virus expressing BgTRAP might be a vaccine candidate against canine *B. gibsoni* infection. Therefore, further study will be required to evaluate the immune response by immunizing dog and develop a more effective vaccine. Recently, it was demonstrated that a heterologous prime-boost immunization regimen with a DNA plasmid and a recombinant vaccinia virus, both expressing the same antigen of pathogens, could induce strong immune responses, including cell-mediated immunity (McShana et al., 2001; Schneider et al., 1999; Sullivan et al., 2000). In mouse malaria, it has been demonstrated that immunization with such a regimen induced complete

protection against sporozoite challenge (Schneider et al., 1998). Therefore, further study need to determine the potential use of such a heterologous immunization regimen with BgTRAP to improve the protective effect of BgTRAP against canine *B. gibsoni* infection.

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Figures

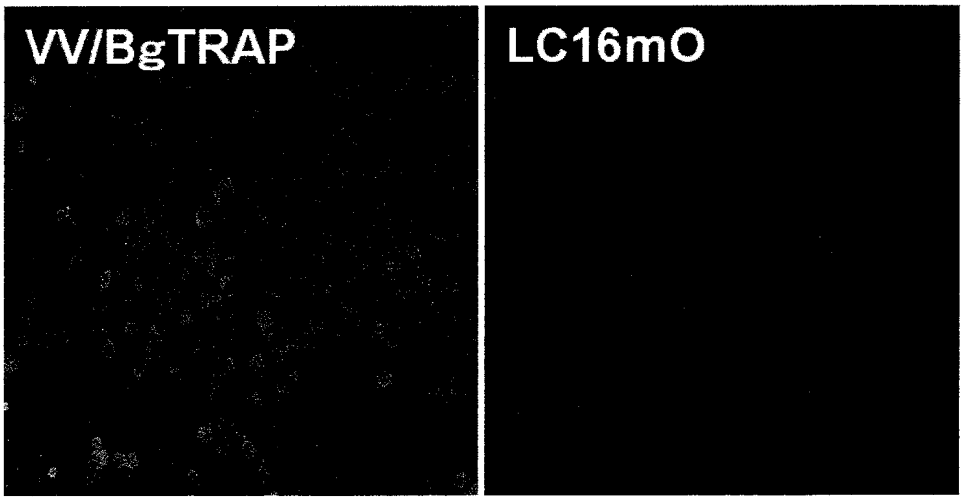

Fig. 1. IFAT analysis of recombinant BgTRAP expressed in RK13 cells by vaccinia virus. VV/BgTRAP, cells infected with VV/BgTRAP; LC16mO, cells infected with control parent LC16mO. The test was performed 48 hrs post-infection with anti-rBgTRAP-specific rabbit serum followed by Alexa Flour-488- conjugated secondary antibodies. Alexa Flour-488-stained proteins are visualized in green.

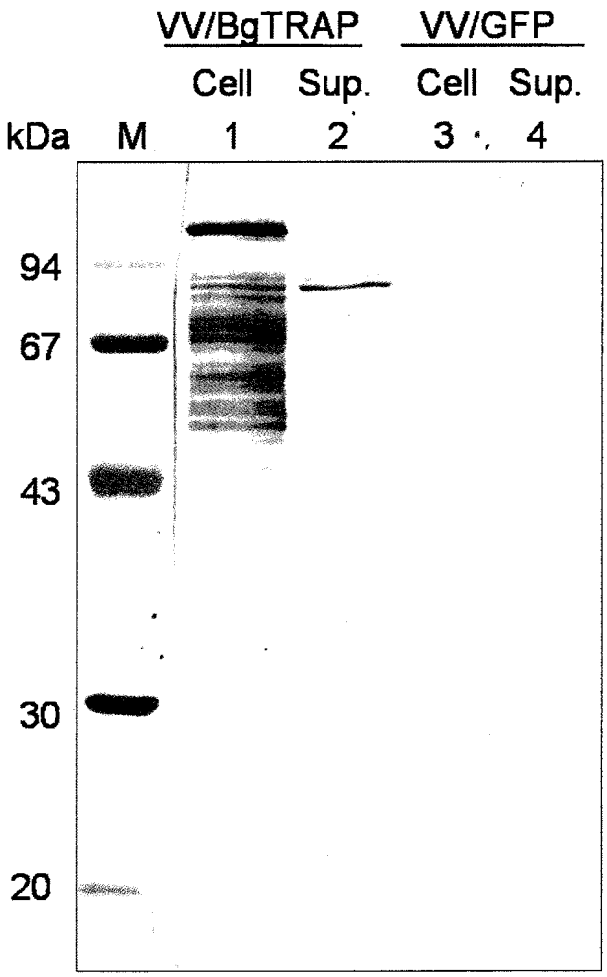

Fig. 2. Western blot analysis of recombinant vaccinia virus using anti-rBgTRAP rabbit serum. Cell lysates of RK13 cells infected with VV/BgTRAP (lane 1), culture supernatants of RK13 cells infected with VV/BgTRAP (lane 2), cell lysates of RK13 cells infected with VV/GFP (lane 3) and culture supernatants of RK13 cells infected with VV/GFP (lane 4) were separated by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using anti-rBgTRAP rabbit serum as the primary antibody. A specific band with molecular mass of about 80 kDa, which was similar to that of native BgTRAP on merozoites of *B. gibsoni*, was detected in the supernatants of VV/BgTRAP-infected RK13 cells. lane M, molecular mass markers.

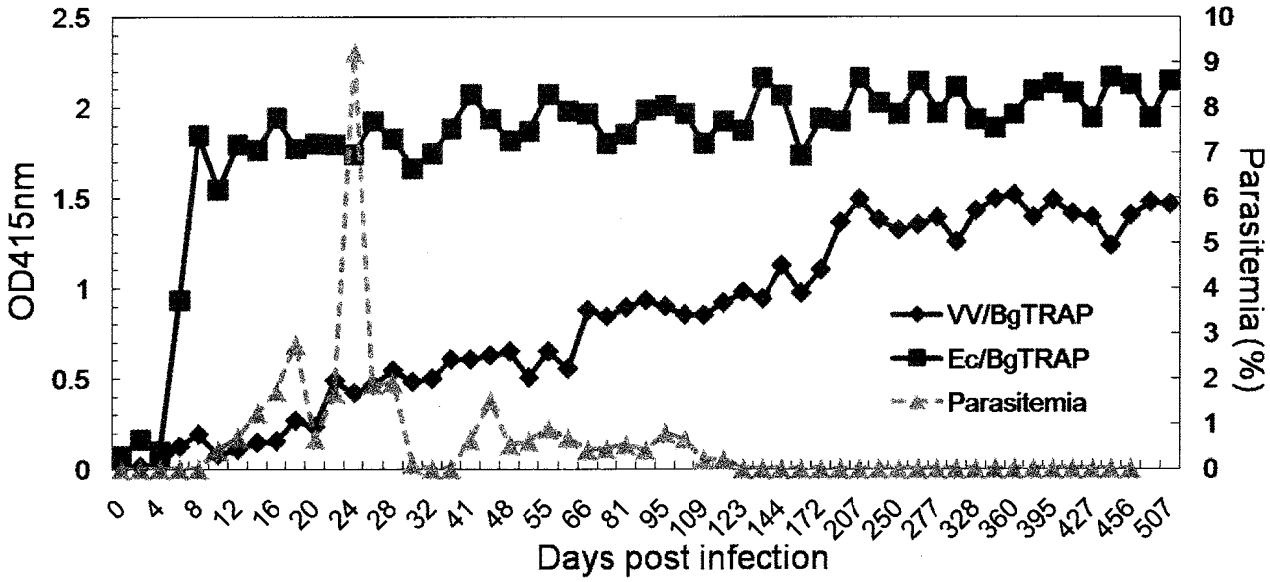

Fig. 3. Antibody response to the VV/BgTRAP culture supernatants antigen in a dog experimentally infected with *B. gibsoni* by ELISA. The parasitemia was determined by microscopic examination. ◇, VV/BgTRAP culture supernatants antigen; □, BgTRAP expressed in *E. coli*; △, Parasitemia.

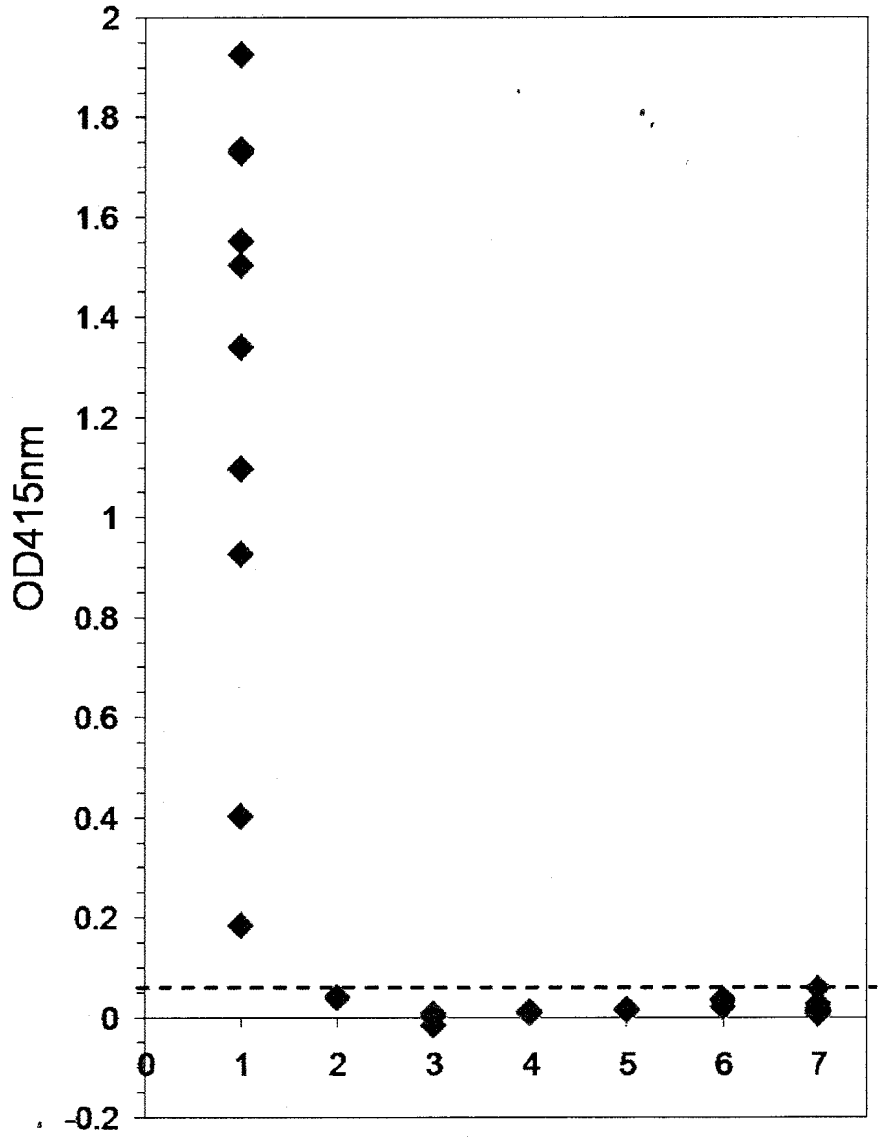

Fig. 4. ELISA with VV/BgTRAP culture supernatants antigen. Lane 1, *B. gibsoni*-infected dog sera (n=10); lane 2, *B. canis vogeli*-infected dog sera (n=2); lane 3, *B. canis rossi*-infected dog sera (n=3); lane 4, *B. canis canis*-infected dog sera (n=3); lane 5, *L. infantum*-infected dog sera (n=3); lane 6, *N. caninam*-infected dog sera (n=3); lane 7, SPF dog sera (n=25).

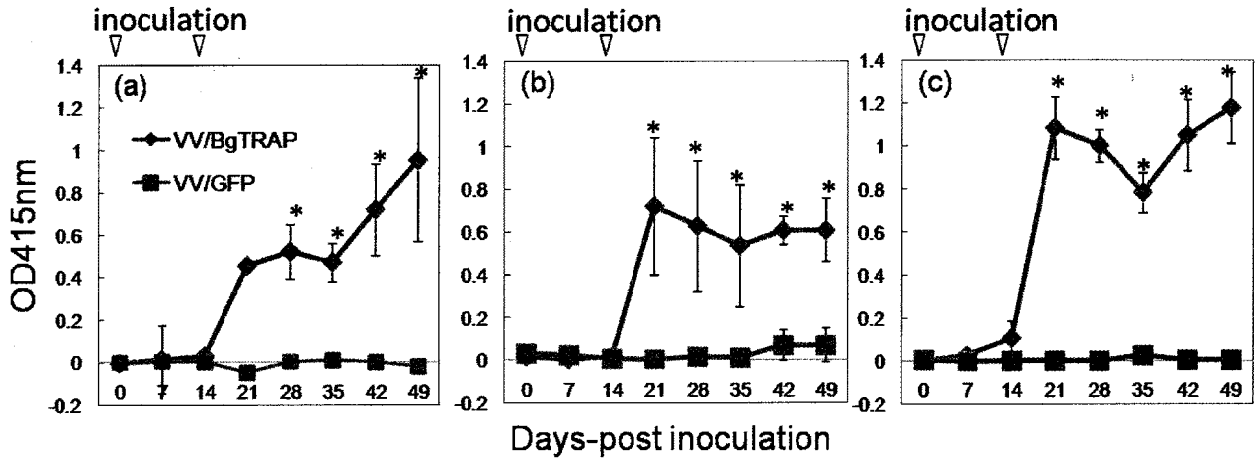

Fig. 5. *B. gibsoni*-specific antibody responses of mice vaccinated with recombinant vaccinia viruses and control mice inoculated with VV/GFP. (a) Total IgG, (b) IgG1, and (c) IgG2a. Mice were inoculated with 1×10^6 pfu of vaccinia viruses at days 0 and 13. Serum samples were collected at 1 week interval from each mouse and *B. gibsoni*-specific antibody responses were measured by ELISA. Antibody titers were expressed as the absorbance at 415 nm. *, The level of antibody vaccinated with VV/BgTRAP was significantly higher than that of control mice ($P<0.05$). ◇, VV/BgTRAP-infected; □ VV/GFP-infected.

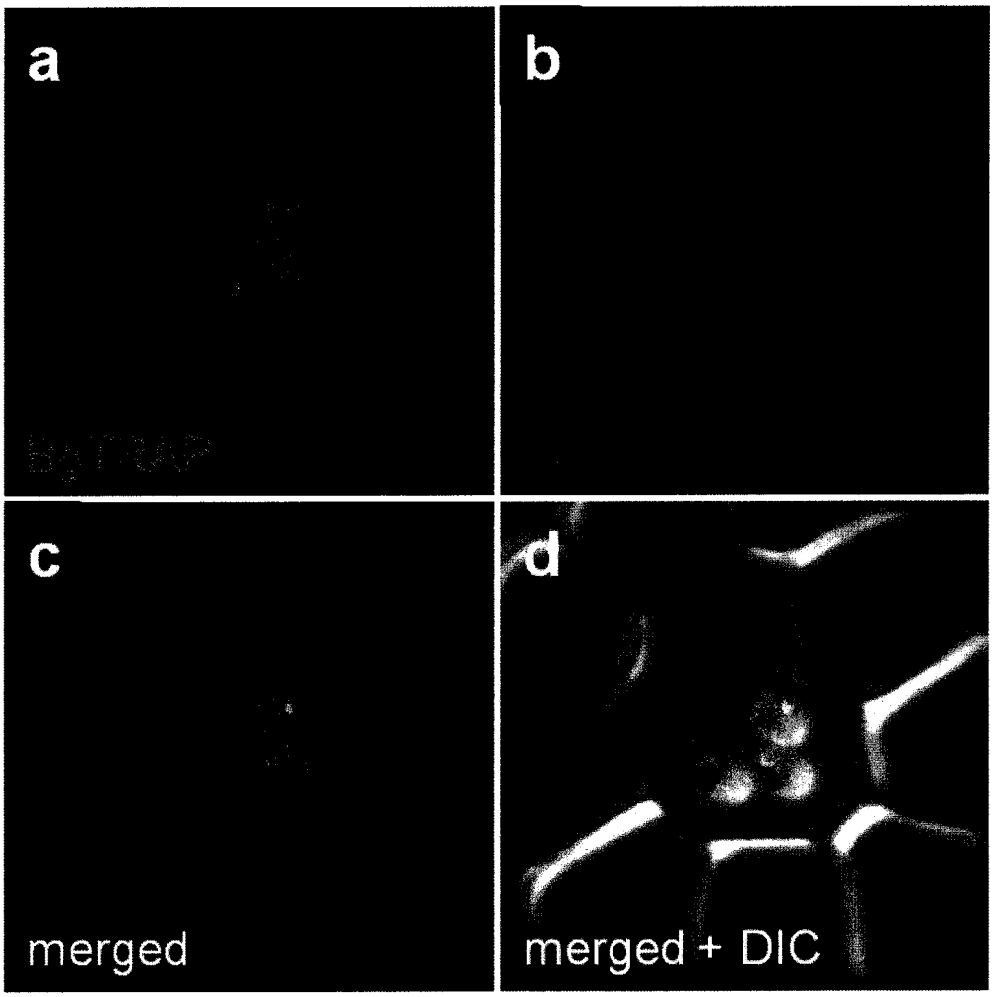

Fig. 6. Detection of the antibody response against *B. gibsoni* antigen using IFAT. (a) Immunofluorescent staining of *B. gibsoni* merozoites with the anti-VV/BgTRAP serum; (b) Propidium Iodide staining of the *B. gibsoni* merozoite nuclei; (c) Overlaid image a and b. (d) Overlaid image of c with phase-contrast image of infected canine erythrocytes. The four images were derived from a single section.

Summary in Japanese

犬バベシア感染症を標的とした組換えワクシニアウイルスベクターの作製とその免疫原性の評価

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【目的】　人類は大昔から多くの感染症の脅威にさらされてきた。医療技術の発展により、感染症に対して、予防的に獲得性免疫を誘導するワクチンが効果的であることが明らかになっている。ワクチンは数多く開発されてきたが、本研究ではその中でも生きた微生物に目的とする病原体遺伝子の一部を人為的に組み込ませ、これを生ワクチンとして利用する「組換えベクターワクチン」に注目した。今回ベクターとして使用したのは、天然痘撲滅に一役買ったワクシニアウイルスである。ウイルスの中では巨大であり、導入できる遺伝子部分も大きく、安全性も評価されているなどの利点から各種感染症に対するワクチンの開発に利用されている。一方、犬バベシア（*Babesia gibsoni*）感染症は、原虫の赤血球内寄生によって引き起こされるマダニ媒介性疾患であり、溶血性貧血を主徴とする臨床症状を示し、病態により死に至ることもある。しかし、有効なワクチンは開発されておらず、本感染症の制圧のための有効な予防法の確立が急がれている。そこで本研究では、*B. gibsoni* の感染防御抗原とされるBgTRAP(*B. gibsoni* thrombospondin-related adhesive protein)を発現する組換えワクシニアウイルスを作製し、その免疫原性の評価を行った。

【方法】　クローニングしたBgTRAP遺伝子を組換えワクシニアウイルス作製用トランスファーベクターに導入した。プラスミドをトランスフェクションした

哺乳動物細胞に弱毒化ワクシニアウイルスを感染させ、薬剤選択によりワクシニアウイルス DNA とプラスミド DNA の間に相同組換えが起こったウイルスを選び出した。以上により BgTRAP を発現する組換えワクシニアウイルス(VV/BgTRAP)を構築した。VV/BgTRAP 発現細胞における BgTRAP の発現と分子量を確認するため、BgTRAP 特異抗体を用いた間接蛍光抗体法と Western blotting 法を行った。さらに、VV/BgTRAP のマウスにおける免疫原性を検討するため、マウスに VV/BgTRAP を 2 回腹腔内接種し、一週間ごとに採血し ELISA により BgTRAP に対する特異抗体の産生を確認した。最後に、*B. gibsoni* を抗原にした間接蛍光抗体法を行うことにより、産生された抗 BgTRAP 抗体が *B. gibsoni* 特異的に反応することを確認した。

【結果】　VV/BgTRAP 感染細胞における BgTRAP の発現が間接蛍光抗体法により認められた。さらに、VV/BgTRAP が発現している BgTRAP は培養上清中に分泌され、*B. gibsoni* 発現の BgTRAP と類似していることを Western blotting 法により確認した。マウスに VV/B g TRAP を腹腔内接種したところ、BgTRAP に対する特異抗体の産生が認められた。得られた抗 BgTRAP 抗体は、実際に *B. gibsoni* 原虫自体にも特異的に反応していることが間接蛍光抗体法により確認された。本研究によって、VV/BgTRAP は動物において原虫特異的抗体を誘導することが明らかになり、そのワクチンとしての効果が期待される。今後、犬における感染防御効果を確かめるためにさらなる研究が必要である。