J. Protozool. Res. 19, 10-23 (2009)

Effect of Oligomannose-Coated Liposome-Based Vaccine on Rodent Babesiosis

Ishigame, T.[1], Takabatake, N.[1], Iseki, H.[1], Ota, N.[1], Koyama, A.[1], Igarashi, I.[1], Nishikawa, Y.[1], Ikehara, Y.[2], Kojima, N.[3] and Yokoyama, N.[1*]

[1]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, [2]Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8568, and [3]Institute of Glycoscience, Tokai University, Hiratsuka, Kanagawa 259-1292, Japan.

[*]Corresponding Author: Naoaki Yokoyama. National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
Tel.: +81-155-49-5649 Fax: +81-155-49-5643 E-mail: yokoyama@obihiro.ac.jp

ABSTRACT

Oligomannose-coated liposome (OML)-based vaccines have been reported to induce Th1-based immunity against entrapped antigens in immunized animals and to show protective effects against several protozoal diseases. In the present study, we produced a recombinant *Babesia rodhaini* ribosomal phosphoprotein P0 (rBrP0) that showed immunological cross reactivity with another rodent *Babesia* parasite, *B. microti*. We evaluated the efficacy of vaccination with OML-entrapped rBrP0 on *B. rodhaini* and *B. microti* infections in mice. Prior immunization with the OML-based rBrP0 vaccine, or with a Freund's adjuvant-based rBrP0 vaccine, failed to demonstrate any protective effect against lethal infection with *B. rodhaini*, but the OML-based vaccine did induce protective immunity against *B. microti* infection, based on a reduction in peak parasitemia levels and prompt clearance of the parasite, compared with control mice. OML might be an effective adjuvant for future vaccines aimed at the control of severe domestic babesioses.

Key words: Oligomannose-coated liposomes (OML); Vaccine; Babesiosis; *Babesia rodhaini*; *Babesia microti*; P0

INTRODUCTION

Babesiosis is a tick-transmitted protozoal infection caused by intraerythrocytic parasites of the genus *Babesia*. *Babesia* parasites infect a wide variety of wild and domestic animals and cause enormous worldwide losses to the livestock industry (Homer *et al.*, 2000). Babesiosis is initiated by tick-borne transmission of the sporozoites, which subsequently invade host red blood cells (RBC) in the infected animals (Yokoyama *et al*., 2006). Merozoites then emerge and reinvade other intact RBC. The asexual reproductive cycle of merozoites induces lysis of the infected RBC, resulting in severe clinical symptoms, such as anemia, fever, and hematuria (Homer *et al.*, 2000). There is currently no safe and effective vaccine for domestic babesioses (Shkap *et al*., 2007). The widespread recognition and severe pathogenesis of bovine and equine *Babesia* parasites have encouraged research into the development of new vaccines to combat babesioses.

There are two species of rodent *Babesia* parasites, *B. rodhaini* and *B. microti*. *B. rodhaini* causes a lethal disease in infected mice (Iseki *et al*., 2008; Kamiya *et al*., 2005), while *B. microti* is characterized by transiently high levels of parasitemia, but subsequent recovery from acute infection in mice (Nishisaka *et al*., 2001; Yokoyama *et al*., 2003). These *Babesia* parasites have been successfully used as experimental models

of infection for other types of babesioses in domestic animals in order to investigate new candidate vaccines and drugs against *Babesia* parasites (Bork *et al*., 2004; Iseki *et al*., 2008; Kamiya *et al*., 2005; Yokoyama *et al*., 2003).

In the present study, we focused on the ribosomal phosphoprotein, P0, which is widely conserved in all eukaryotes (Goswami *et al*., 1996). P0 is related to the family of acidic ribosomal phosphoproteins (Rich and Steitz, 1987) that, together with other ribosomal proteins, P1 and P2, constitute a complex of ribosomal phosphoproteins (Rich and Steitz, 1987). Among these three proteins, only P0 has been reported to be vital for eukaryotic replication, as demonstrated by knockout studies in *Saccharomyces cerevisiae* (Santos and Ballesta, 1994). In *Plasmodium falciparum*, however, the protozoan P0 appears on the surface of parasites in both the sexual and asexual stages (Chatterjee *et al*., 2000a). Anti-*P. falciparum* P0 antibodies inhibited the growth of parasites *in vitro*, and P0 protected immunized mice from *P. yoelii* infection *in vivo* (Chatterjee *et al*., 2000b; Goswami *et al*., 1997). *B. gibsoni* P0 (BgP0) has also been reported to serve as an effective vaccine (Terkawi *et al*., 2007, 2008). Anti-BgP0 antibodies reduced the *in vitro* growth of *B. bovis*, while mice immunized with BgP0, together with Freund's adjuvant, developed protective immunity against *B. microti*. These studies indicate that the protozoan P0 antigens are highly conserved among this related family of protozoa, and can serve as efficient candidate vaccines.

Oligomannose-coated liposomes (OML) provide a safe immune adjuvant, and no skin damage at the injection site has been reported (Fukasawa *et al*., 1998). A previous study showed that OML, coated with a neoglycolipid consisting of mannotriose and dipalmitoylphosphatidylethanolamine (Man3-DPPE), were specifically and rapidly incorporated into intraperitoneal macrophages (Shimizu *et al*., 2007) and accumulated in nearby lymphoid tissues (Ikehara and Kojima, 2007; Kojima *et al*., 2008). Peritoneal macrophages activated by OML expressed MHC class α and co-stimulatory molecules on their surfaces and produced interleukin (IL)-12 (Takagi *et al*., 2007). These results indicate that OML can induce Th1-based immunity against the entrapped antigen in immunized animals. The efficacy of OML as a vaccine adjuvant has already been confirmed in *Leishmania major* (Shimizu *et al*., 2003, 2007) and *Toxoplasma gondii* infections (Kuboki *et al*., 2007), and in tumors (Kojima *et al*., 2008).

In the present study, we isolated the *B. rodhaini* P0 (BrP0) gene and determined that the antigen showed immunological cross-reactivity with *B. microti* P0. We also prepared the purified recombinant protein, designated as rBrP0, and evaluated the effects of vaccination with OML-entrapped rBrP0 on *B. rodhaini* and *B. microti* infections in mice.

MATERIALS AND METHODS

Parasites and mice

The Munich and Australian strains of *B. microti* and *B. rodhaini*, respectively, were maintained by blood passage in mice (Nishisaka *et al*., 2001; Kamiya *et al*., 2005; Iseki *et al*., 2008). Female BALB/c mice were purchased from Clea Japan (Tokyo, Japan). All mice used in the present study were cared for in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (Obihiro, Japan).

Cloning of the BrP0 gene

A DNA fragment encoding the BrP0 gene was amplified from cDNA extracts derived from *B. rodhaini*-infected RBC (Fujii *et al*., 2002) by polymerase chain reaction (PCR). The primers used were: forward primer (5'-GCCGGAGCTCATGCCAAATCCTGTTAAG-3') and reverse primer (5'-GCCGTCTAGATTAGTCAAATAGAGAGAAGCC-3'), which contain *Sac*I and *Xba*I cleavage sites,

respectively (underlined), near their 5' ends. PCR was performed in 50 μ1 of reaction mixture containing 15 pmol of each primer, 100 ng of template DNA, 200 μM of a mixture of deoxynucleoside triphosphates, 10 μl of a 5 x PrimeSTAR buffer (Mg^{2+} plus), and 1.25 U of PrimeSTAR HS DNA Polymerase (TaKaRa Bio, Shiga, Japan). The PCR amplification was carried out for 30 cycles under the following conditions: 10 s at 98°C for denaturation, 10 s at 45°C for annealing, and 1 min at 72°C for extension. The PCR product was digested with *Sac*I and *Xba*I, purified using a MinElute gel extraction kit (Qiagen, Valencia, CA, USA), and then inserted into the *Sac*I and *Xba*I sites of a pCold-TF expression vector (TaKaRa Bio). The nucleotide sequence of the inserted fragment was determined using a Big Dye Terminator kit (Applied Biosystems Japan, Ltd., Tokyo, Japan) with an automated DNA sequencer (ABI PRISM 3100 genetic analyzer, Applied Biosystems Japan, Ltd.) (GenBank accession number AB469168). A CLUSTAL W program (European Bioinformatics Institute, Cambridge, UK) was used to align the putative amino acid sequence of BrP0 with those of other apicomplexan parasite P0 protein sequences. A neighbor-joining method using an NJplot program (Perriere and Gouy, 1996) was used to construct a phylogenetic tree for the P0 proteins.

Preparation of a recombinant BrP0 protein

The cloned BrP0 gene was expressed in *Escherichia coli* (DH-α strain) as a fusion protein with trigger factor (TF) and a His-Tag sequence (Qing *et al.,* 2004). The recombinant protein was then purified from the culture extract using Ni-NTA agarose beads (Qiagen) according to the manufacturer's instructions. The clarified product was dialyzed against phosphate-buffered saline (PBS) at 4˚C overnight, and designated as rBrP0. The concentration of rBrP0 was measured using a Lowry protein assay kit (Pierce, Rockford, IL, USA)

Production of mouse anti-rBrP0 antibodies

Five female BALB/c mice were immunized three times at 2-week intervals with the purified rBrP0 antigen to produce anti-rBrP0 antibodies. First, the mice were inoculated subcutaneously (s.c.) with 50 μg rBrP0 emulsified with complete Freund's adjuvant (Difco Lab., Detroit, MI, USA). The second and third immunizations were performed using 25 μg rBrP0 with incomplete Freund's adjuvant (Difco Lab.). Sera were collected from the immunized mice on day 10 after the last immunization.

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

The rBrP0 (BrP0-TF) and control TF were mixed at 1:1 (v/v) with 2 x SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue), and boiled for 3 min. The samples were then either electrophoresed by SDS-PAGE (e-PAGEL® E-R1020L, ATTO, Tokyo, Japan), and the gel was stained with Coomassie brilliant blue (CBB), or were subjected to Western blot analysis. For Western blotting, the proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was washed twice with 0.1% Tween-20 in PBS (PBST), and blocked in 5% skimmed milk in PBST at 4˚C, overnight. The membrane was incubated with *B. microti*-infected mouse serum diluted at 1:250 (v/v) with PBST for 1 h at room temperature (RT), and then washed with PBST. The membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobin G (IgG) (DAKO, Produktionsvej, Denmark) diluted at 1:1,500 with PBST for 1 h at RT. Visualization of the antigen-antibody complexes was performed by subsequent application of diaminobenzidine (Wako Pure Chemical Industries, Osaka, Japan) with H_2O_2.

Indirect fluorescent antibody test (IFAT)

When the level of parasitemia exceeded 30%, *B. rodhaini*- or *B. microti*-infected RBC were prepared from the tail veins of infected mice, and washed three times with PBS. The infected RBC were coated onto IFAT slides (Matsunami, Osaka, Japan), dried completely, and then fixed in an acetone-methanol mixture at

1:1 (v/v) at -20°C for 3 min. Mouse anti-rBrP0 antibody was applied to the fixed smears as the first antibody (1:300), and they were then incubated for 1 h at RT. After washing three times with PBS, Alexa-Fluor® 488-conjugated goat anti-mouse IgG (DAKO) was applied as the secondary antibody (1:2,000), and the slides were then incubated for 1 h at RT. After washing three times with PBS, the slides were incubated with 250 μl/ml propidium iodide (Wako Pure Chemical Industries) and Cytomarion Fluoresent Mounting Medium (DAKO) mixed at 1:1 (v/v), for 1 h at RT. The slides were finally viewed under a confocal laser-scanning microscope (TCS NT, Leica, Wetziar, Germany) to detect the fluorescent signals of the antigen-antibody complexes, as well as the parasite nuclei (Kamiya *et al*., 2005).

Preparation of liposomes

OML were prepared as described previously (Shimizu *et al*., 2007; Kuboki *et al*., 2007; Kojima *et al*., 2008). Briefly, a chloroform-methanol solution at 2:1 (v/v) containing 1.5 μmol of dipalmitoylphosphatidylethanolamine (NOF Corporation, Tokyo, Japan) and 1.5 μmol of cholesterol (Sigma, Tokyo, Japan) was placed in a conical flask, and then dried by rotary evaporation. Subsequently, 2 ml of chloroform containing 0.15 μmol of Man3-DPPE (Mizuochi *et al*., 1989) was added to the flask, and then evaporated to prepare a lipid film containing the neoglycolipid. Two hundred microliters of PBS containing the indicated recombinant protein at 500 μg/ml were added to the dried lipid film, and multilamellar vesicles were prepared by intense vortex dispersion. The multilamellar vesicles were extruded 10 times through a 1-μm pore polycarbonate membrane (Nucleopore, Pleasanton, CA, USA). Liposomes entrapping the recombinant protein were separated from free recombinant protein by three successive rounds of washing with PBS and centrifugation at 20,000 × *g* for 30 min at 4°C. The amount of entrapped antigen was measured using a modified Lowry protein assay reagent (Pierce) in the presence of 0.3% (w/v) SDS.

Mouse experiments

Experiment 1: A total of 24 female BALB/c mice were divided into four groups. All mice were 7 weeks old at the first immunization. The four groups of mice (n=6 each) were inoculated intraperitoneally (i.p.) with 100 μg of rBrP0 or TF emulsified with complete Freund's adjuvant, complete Freund's adjuvant alone, or PBS alone. Booster immunizations using incomplete Freund's adjuvant were performed on days 14 and 24 after the first immunization. At 7 days after the third immunization, all mice were challenged i.p. with 1×10^6 *B. rodhaini*-infected RBC. After infection, thin blood smears were periodically prepared for subsequent Giemsa-staining. Parasitemia dynamics and survival rates were monitored in all groups, as described previously (Kamiya *et al*., 2005; Iseki *et al*., 2008).

Experiment 2: A total of 35 female BALB/c mice were divided into five groups. Mice were 8 weeks old at the first immunization. Mice in the five groups (n=7 each) were inoculated s.c. with 3 μg rBrP0 entrapped in OML (rBrP0-OML), 3 μg TF entrapped in OML (TF-OML), 3 μg rBrP0 or TF in PBS, or PBS alone (200 μl each). Booster immunizations were administered via the same route on day 7 after the first immunization. At 7 days after the second immunization, all mice were challenged i.p. with 1×10^7 *B. rodhaini*-infected RBC. After the challenge infection, parasitemia dynamics and survival rates were monitored in all groups until the death of the mice.

Experiment 3: A total of 42 female BALB/c mice were divided into six groups. Mice were 8 weeks old at the first immunization. Mice in the six groups (n=7 each) were inoculated s.c. with 3 μg rBrP0 entrapped in OML (rBrP0-OML), 3 μg TF entrapped in OML (TF-OML), no antigen-entrapped OML (PBS-OML), 3 μg rBrP0, TF in PBS, or PBS alone (200 μl each), respectively. The amount of "PBS-OML" inoculated in the indicated group was adjusted to be the same as that in "rBrP0-OML" group, based on the cholesterol concentration. Booster immunizations were administered via the same route on day 7 after the first

immunization. At 7 days after the second immunization, all mice were challenged i.p. with 1×10^7 *B. microti*-infected RBC. After the challenge infection, Giemsa-stained thin blood smears were prepared from the tail veins of mice every 2 (days 2–18) or 3 days (days 21–36), and the parasitemia dynamics were monitored in all groups for a total of 36 days. Serum fractions were prepared from all mice immediately before immunization and infection, and on day 4 after infection, for cytokine analyses.

Cytokine analyses

IL-2, IL-4, IL-10, IL-12p70, and gamma-interferon (IFN-γ) levels in the collected sera were measured using mice Th1/Th2 cytokine and inflammatory cytometric bead array kits (BD Biosciences Pharmingen, San Diego, CA, USA) with an FACSCalibur flow cytometer (BD Biosciences Pharmingen), as described previously (Iseki *et al*., 2008).

Statistical analyses

The survival rates of *B. rodhaini*-infected mice were plotted according to the Kaplan-Meier method and differences in the survival rates among the groups were analyzed using the generated Wilcoxon test. Differences in parasitemia and cytokine concentrations among all groups were analyzed using independent Student's *t*-tests. Differences were accepted as significant at a level of $P < 0.05$.

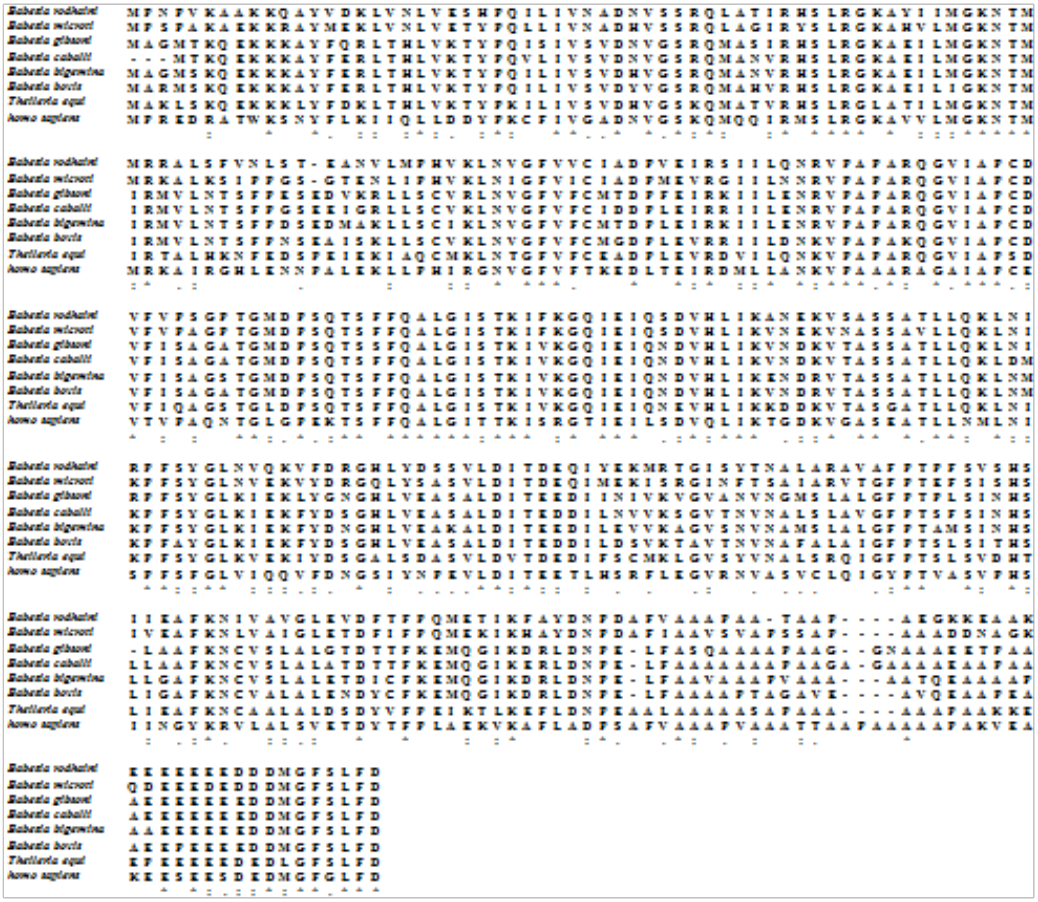
RESULTS

Structural and immunogenic analyses of BrP0

A cDNA clone containing the BrP0 gene had been previously obtained from a *B. rodhaini* cDNA expression phage library in an immunoscreening experiment with *B. microti*-infected mouse serum (Takabatake *et al*., in prep.). Therefore, the protein, designated as BrP0 in the present study was expected to be a common immunogenic antigen that could be recognized by both *B. rodhaini*- and *B. microti*-infected mouse sera. CLUSTAL W analysis also showed that the amino acid sequence of BrP0 (GenBank accession number AB469168) had high sequence homology with ribosomal phosphoproteins from other *Babesia* parasites: *B. microti* (74%), *B. caballi* (60%), *B. bigemina* (59%), *Theileria equi* (59%), *B. bovis* (59%), and *B. gibsoni* (58%), as shown in Figure 1A. In the phylogenetic tree of P0 proteins, BrP0 is located relatively near to *B. microti* P0, compared with the locations of other *Babesia* P0 proteins (Fig. 1B). P0 proteins are therefore highly conserved in their sequences among the family of *Babesia* (and *Theileria*) parasites.

The recombinant protein, rBrP0, and the tag-control, TF protein, were successfully produced as highly soluble forms in *E. coli* (data not shown), and purified using Ni-NTA agarose beads. These proteins were detected with molecular masses of 87 and 48 kDa, respectively, using SDS-PAGE and CBB staining (Fig. 2A). In order to confirm the cross-reactivity of BrP0 against *B. microti*-infected mouse serum, Western blot analyses of the rBrP0 and TF antigens were carried out; the *B. microti*-infected serum reacted with the 87-kDa rBrP0, but not with the 48-kDa TF (Fig. 2B, left panel). No signal was detectable when non-infected control mouse serum was used as the first antibody (Fig. 2B, right panel). Additionally, mouse anti-rBrP0 antibodies recognized both *B. rodhaini* and *B. microti* antibodies, although the reactivity to *B. microti* was weaker than that to *B. rodhaini* (Fig. 3). In contrast, control mouse serum showed no reaction with either parasite (data not shown).

A



B

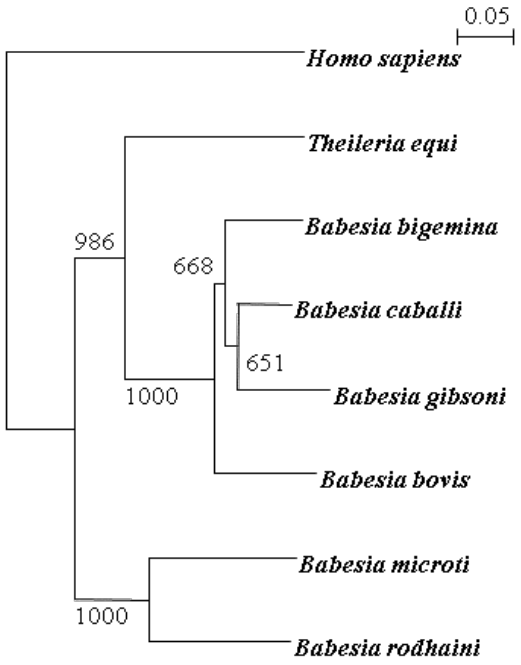


Fig. 1. Sequence analyses of *B. rodhaini* ribosomal phosphoprotein (P0). Panel A. Multiple-sequence alignments of the deduced amino acid sequence of *B. rodhaini* P0 with those of other *Babesia* (*Theileria*) parasites. Amino acid gaps and identities are indicated by dashes and asterisks, respectively, while similarities are shown by colons or dots. Panel B. Phylogenetic tree of P0 proteins. The numbers shown at the branch nodes indicate the bootstrap values. GenBank accession numbers are: *Homo sapiens* (NP444505), *B. rodhaini* (AB469168), *B. gibsoni* (ACA14449), *B. caballi* (BAF91359), *T. equi* (BAF91358), *B. bigemina* (BAF91357), *B. bovis* (AAM18123), and *B. microti* (BAF45856).

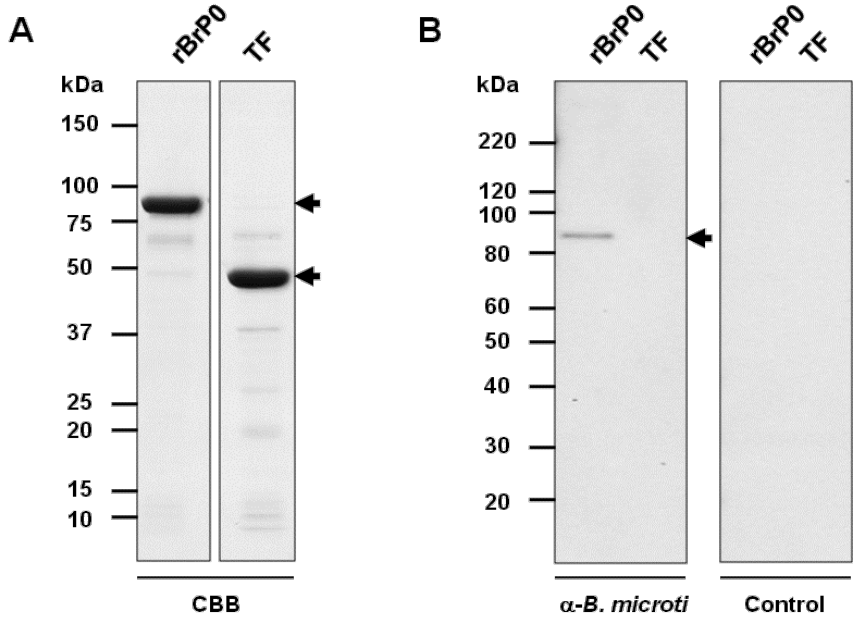


Fig. 2. Production of recombinant *B. rodhaini* P0 (rBrP0) and cross-reactivity of *B. microti*-infected mouse serum with rBrP0. Panel A: The purified recombinant proteins (rBrP0 and TF) were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining. Molecular mass standards are indicated in kDa on the left. Arrows indicate the bands of rBrP0 and TF. Panel B: Immunoreactivity of rBrP0 and TF were observed by Western blot analysis with *B. microti*-infected (α-*B. microti*) or non-infected mouse serum (control). Molecular mass standards are indicated in kDa on the left. An arrow indicates the band of rBrP0 detected with *B. microti*-infected mouse serum.

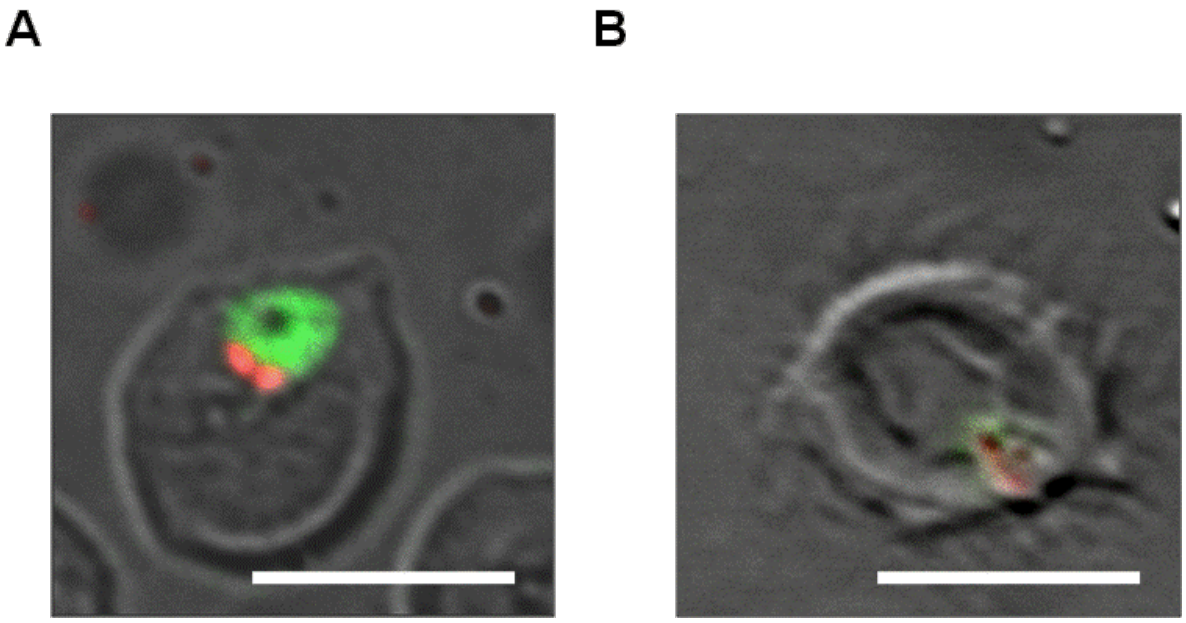


Fig. 3. Indirect fluorescent antibody test using mouse anti-rBrP0 antibody on smears of *B. rodhaini*- (Panel A) and *B. microti*-infected RBC (Panel B). Bar = 5 μm. Note: the anti-rBrP0 antibody reacted to both *B. rodhaini* and *B. microti* antibodies, although the reactivity of *B. microti* was weaker than that of *B. rodhaini*.

Effect of Freund's adjuvant-based rBrP0 vaccine on *B. rodhaini* infection

One group of mice (n=6) received three immunizations with rBrP0 in Freund's adjuvant before challenge with 1×10^6 *B. rodhaini*-infected RBC. The parasitemia dynamics and the survival rate of this group were compared with those of the other control groups, as shown in Figures 4A and 4B, respectively. All mice, except for one mouse immunized with TF alone, showed the development of typical parasitemia, and had died by day 11 after infection. There were no differences in the levels of parasitemia and survival rates between the rBrP0-immunized group and all of the other control groups. Freund's adjuvant-based rBrP0 vaccine was therefore ineffective against *B. rodhaini* infection in mice.

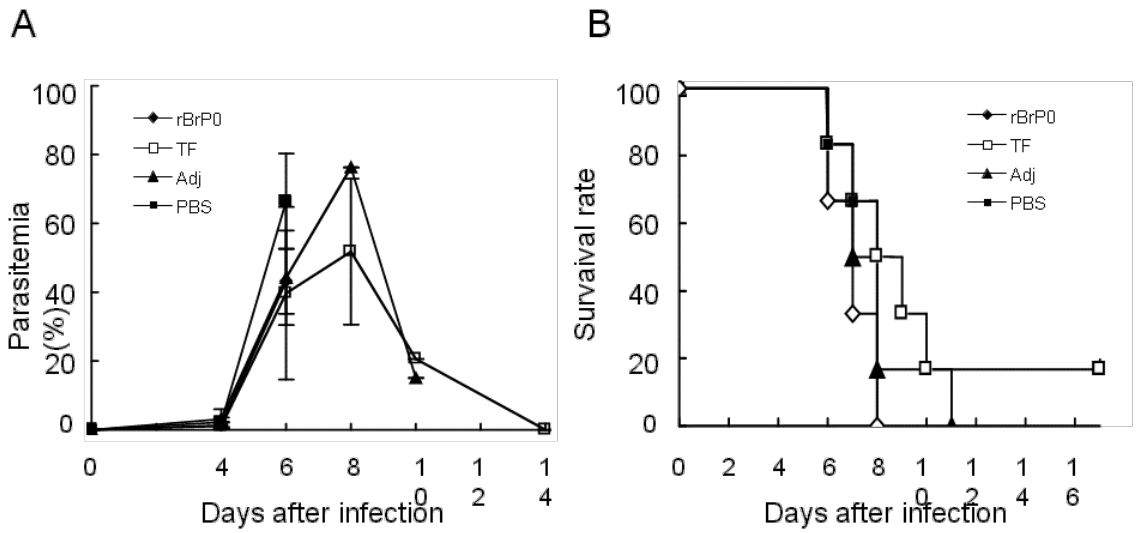


Fig. 4. Effect of Freund's adjuvant-based rBrP0 vaccine on *B. rodhaini* infection. Mean parasitemia (Panel A) and survival rates (Panel B) of *B. rodhaini*-infected mice are indicated in each group. Mice were immunized once with complete Freund's adjuvant, and then boosted twice with incomplete Freund's adjuvant. Groups were immunized with rBrP0 (filled diamonds), TF (open boxes), adjuvant alone (filled triangles), or PBS alone (filled boxes). Bars indicate the mean ± standard deviation (SD). Note no significant difference among all groups.

Effects of OML-based rBrP0 vaccine on *B. rodhaini* and *B. microti* infections

The effects of an OML-based vaccine entrapping rBrP0 were evaluated against *B. rodhaini* and *B. microti* infections in experiments 2 and 3, respectively. In experiment 2 (*B. rodhaini* infection), each group consisted of six mice, but one mouse in the rBrP0 alone-immunized group accidentally died before infection; the data for this mouse were therefore excluded. The results of experiment 2 showed that the level of parasitemia increased rapidly in all groups, and all mice were dead by day 6 after infection with *B. rodhaini*, as shown in Figures 4A and 4B. However, in experiment 3 (*B. microti* infection, which is not lethal in mice), there was a significant difference in the dynamics of parasitemia between the "rBrP0-OML" and "TF-OML" groups, as shown in Figure 5. The "rBrP0-OML" immunized group showed a low peak of parasitemia on day 8 ($P = 0.022$) and a prompt reduction in the number of circulating parasites from day 10 ($P = 0.022$) after infection, compared with the parasitemia in the "TF-OML" immunized group. Additionally, when the level of parasitemia in the PBS control group was compared with those in other groups, only the "rBrP0-OML" group showed a significantly lower level of parasitemia on day 10 ($P = 0.009$) and days 12–21 ($P < 0.05$) after infection (Fig. 6). The OML-based rBrP0 vaccine therefore induced protective immunity against *B. microti* infection, but not *B. rodhaini* infection, in mice.

In order to evaluate the host immune response to *B. microti* infection in the rBrP0-OML group, five cytokines (IL-2, IL-4, IL-10, IL-12p70, and IFN-γ) were measured in the serum fractions collected from the mice immediately before inoculation (no treatment and no infection), and on days 0 and 4 after infection in experiment 3 (Table 1). The average concentrations of serum cytokines before treatment were set as baselines (value 1.0) in each group, and the subsequent changes in cytokine levels on day 0, immediately before infection, and on day 4 after infection are shown in Figure 7. On day 0, all groups had serum cytokine levels within the normal range (Fig. 7A). In the OML-immunized groups (rBrP0-OML, TF-OML, and PBS-OML), however, Th1 cytokines, including IL-2, IFN-γ, and IL-12p70, tended to be up-regulated on day 4, although the differences between the baseline and day-4 levels were not significant. On the other hand, levels of Th2 cytokines, including IL-4 and IL-10, on day 4 were lower in the "rBrP0-OML" immunized group, compared with other groups.

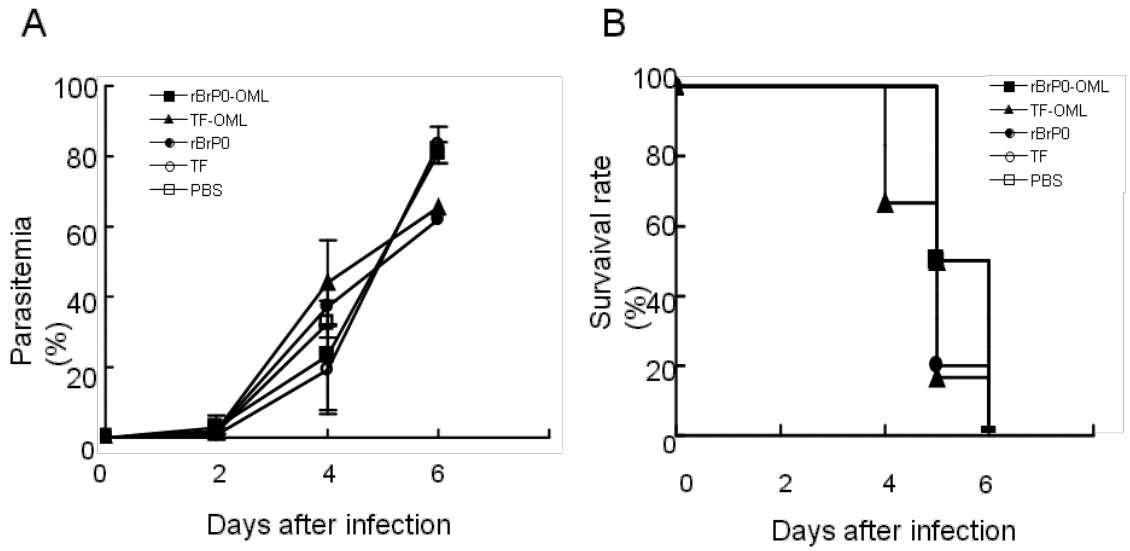


Fig. 5. Effect of OML-based rBrP0 vaccine on *B. rodhaini* infection. Mean parasitemia (Panel A) and survival rates (Panel B) of *B. rodhaini*-infected mice are indicated in each group. Groups were immunized with rBrP0-OML (filled boxes), TF-OML (triangles), rBrP0 (filled circles), TF (open circles), or PBS alone (open boxes). Bar indicate the mean ± SD. Note no significant difference among all groups.

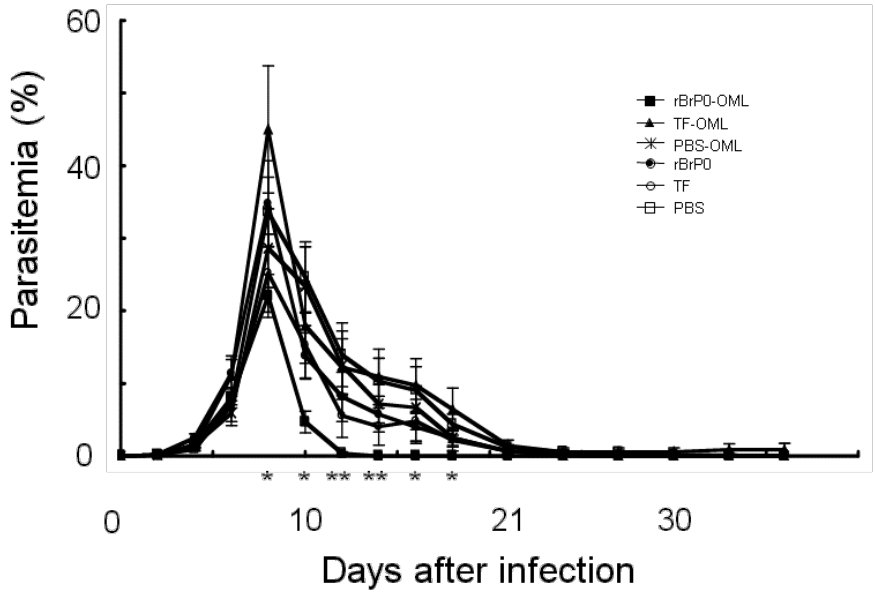


Fig. 6. Effect of OML-based rBrP0 vaccine on *B. microti* infection. Dynamics of mean parasitemia of *B. microti*-infected mice are indicated in each group. Groups were immunized with rBrP0-OML (filled boxes), TF-OML (filled triangles), PBS-OML (crosses), rBrP0 (filled circles), TF (open circles), or PBS alone (open boxes). Bars indicate the mean ± standard error (SE). The asterisks indicate significant differences between "rBrP0-OML" and "TF-OML" immunized groups (*P>0.05, **P>0.01).

Table 1. Average concentration (pg/ml) of serum cytokines in the *B. microti*-infected mice.

	rBrP0-OML	TF-OML	PBS-OML	rBrP0	TF	PBS
(IFN-γ)						
no treatment	9.02 ±2.93	10.88 ±0.53	15.66 ±0.34	17.34 ±0.49	19.25 ±0.69	22.51 ±0.82
day0	12.99 ±2.24	12.46 ±0.68	17.02 ±0.75	17.98 ±0.90	17.67 ±0.68	20.78 ±0.98
day4	40.04 ±5.02	47.56 ±6.52	36.33 ±3.35	49.23 ±5.76	41.65 ±4.25	36.59 ±3.80
(IL-2)						
no treatment	12.49 ±3.43	14.70 ±1.45	18.80 ±0.78	25.05 ±0.98	27.63 ±0.71	33.61 ±1.14
day0	14.90 ±2.69	10.91 ±3.05	22.80 ±1.25	24.96 ±1.73	23.61 ±3.05	31.68 ±1.55
day4	16.42 ±1.35	17.61 ±1.58	22.46 ±1.09	23.12 ±1.12	26.23 ±0.78	24.21 ±1.95
(IL-12p70)						
no treatment	39.29 ±5.92	32.55 ±1.41	48.48 ±1.10	88.43 ±0.90	104.57 ±1.61	148.51 ±1.10
day0	36.31 ±5.42	31.44 ±6.15	82.52 ±7.68	89.41 ±11.52	81.90 ±8.12	128.23 ±9.03
day4	57.71 ±12.10	55.58 ±8.39	80.35 ±10.12	91.78 ±7.64	119.75 ±5.17	98.59 ±16.78
(Il-4)						
no treatment	27.83 ±30.85	11.57 ±7.46	18.90 ±6.00	27.23 ±5.82	28.98 ±9.91	34.38 ±8.00
day0	23.16 ±4.25	12.03 ±3.37	18.78 ±1.40	26.42 ±2.11	24.98 ±6.15	32.76 ±1.33
day4	17.63 ±3.42	16.53 ±4.48	28.80 ±2.40	32.53 ±2.70	36.63 ±1.16	30.20 ±6.04
(IL-10)						
no treatment	164.55 ±11.30	25.53 ±4.92	91.24 ±4.90	102.46 ±3.25	114.93 ±7.22	187.16 ±6.93
day0	186.62 ±33.18	36.07 ±8.12	102.04 ±9.53	118.52 ±15.79	100.51 ±3.37	170.45 ±17.09
day4	164.52 ±24.27	182.23 ±65.44	348.08 ±44.56	360.17 ±50.16	514.19 ±23.72	424.18 ±77.03

Serum fraction was prepared from blood collected from tail vein of all mice.
Each value indicates the mean ± SE.

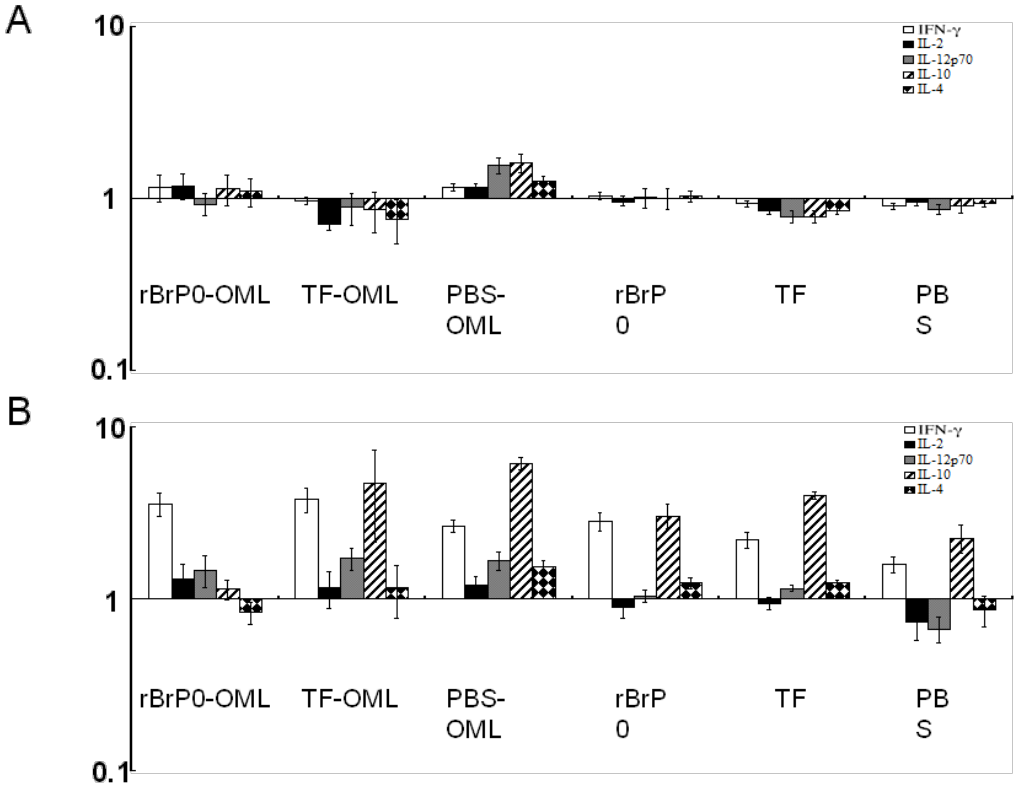


Fig. 7. Relative dynamics of serum cytokine levels on days 0 (Panel A) and 4 (Panel B) after *B. microti* infection (Experiment 3). All average concentrations of serum cytokines (IL-2, IL-4, IL-10, IL-12p70, and IFN-γ) before treatment were set as baselines to 1.0 in each group, and subsequent dynamics of the cytokines were expressed as relative values. Bars indicate the mean ± SE.

DISCUSSION

In the present study, we demonstrated that BrP0 was structurally similar to *B. microti* P0 and that the recombinant antigen (rBrP0) was immunologically recognized by both *B. rodhaini*- and *B. microti*-infected mouse sera. These results suggested that rBrP0 could be used as a common vaccine antigen against both the rodent *Babesia* infections.

We found no protective effect of vaccination with rBrP0 emulsified with Freund's adjuvant on lethal infection by *B. rodhaini*. An OML-based vaccine has been shown to induce a Th1 immune response specific for the entrapped antigen in immunized mice (Ikehara *et al*., 2007), and we therefore tested the effect of immunization with rBrP0-OML. However, contrary to our expectations, the lethal growth of *B. rodhaini* was not controlled by rBrP0-OML immunization. rBrP0-OML-immunized mice did, however, show significantly lower levels of parasitemia after *B. microti* infection, compared with TF-OML and PBS-OML control mice, indicating that OML-entrapped rBrP0 was able to induce protective immunity against *B. microti* infection.

Igarashi *et al*. (1999) previously reported that IFN-γ-producing $CD4^+$ T cells played an important role in the prevention of acute infection by *B. microti*, and acted mainly before the peak level of parasitemia was reached. We therefore expected that the development of *B. microti* parasitemia would be regulated in the rBrP0-OML immunized group before the peak level of parasitemia occurred in control mice (day 8). However, the reduction in parasitemia was mainly observed between days 8 and 18 after infection. The high

injected dose (1×10^7) of infected RBC might have masked the inhibitory effect of rBrP0-OML immunization on parasite growth in the acute phase, and further studies are needed to clarify the rBrP0-OML-generated immune response against *B. microti* infection. In contrast, Shimada *et al*. (1996) reported that the host immune response against *B. rodhaini* infection was entirely different from that against *B. microti,* and that protective immunity against *B. rodhaini* infection may not involve IFN-γ-producing $CD4^+$ T cells. Based on these previous results, the immunity induced by an OML-based vaccine might not be effective against *B. rodhaini* infection.

In order to evaluate the immune response against *B. microti* infection generated by rBrPO-OML, we measured serum cytokine levels. On day 4 after infection, all OML-immunized groups exhibited higher concentrations of Th1 cytokines (IL-2, IFN-γ, and IL-12), compared with those in the non-OML immunized groups. These results suggest that OML alone could up-regulate Th1-related immunity after *B. microti* infection. However, only the rBrP0-OML immunized group failed to show an increase in Th2 cytokine levels (IL-4 and IL-10) after infection, compared with the other groups that showed relatively high concentrations of Th2 cytokines on day 4. Because IL-4 and IL-10 play important roles in switching from Th1 to Th2 immune responses in the host (Reiner, 1994; Sher and Coffman, 1992; Mosmann and Moore, 1991), increases in IL-4 and IL-10 levels in all groups, except for the rBrP0-OML group, might lead to a switch to a Th2-mediated immune response against *B. microti* infection, resulting in a failure of vaccine efficacy. The cytokine levels suggest that only OML-entrapped rBrP0 was able to induce a dominant Th1-mediated immune response against parasite infection in the immunized mice.

Because the phosphoprotein P0 is widely conserved among the *Babesia* family, this parasite's P0 could act as a novel vaccine against many other babesioses. Bovine babesiosis in particular, which is an economically important tick-borne disease of cattle in tropical and subtropical regions of the world (Homer *et al*., 2000), urgently requires an effective vaccine and use of an OML-based vaccine should be considered in this situation. An OML-based vaccine system might also be applicable to many other serious pathogens that require a Th1-mediated immune response for their eradication.

In conclusion, the OML-based rBrP0 vaccine constructed in the present study induced protective immunity against *B. microti* infection in mice. OML-based vaccines could become powerful tools against several pathogenic infections, including severe domestic babesiosis.

ACKNOWLEDGMENTS

This study was supported by Grants from the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), Japan.

REFERENCES

Bork, S., Yokoyama, N., Ikehara, Y., Kumar, S., Sugimoto, C. and Igarashi, I. 2004. Growth-inhibitory effect of heparin on *Babesia* parasites. Antimicrob. Agents. Chemother. 48:236-241.

Chatterjee, S., Singh, S., Sohoni, R., Kattige, V., Deshpande, C., Chiplunkar, S., Kumar, N. and Sharma, S. 2000a. Characterization of domains of the phosphoriboprotein P0 of *Plasmodium falciparum.* Mol. Biochem. Parasitol. 107:143-154.

Chatterjee, S., Singh, S., Sohoni, R., Singh, N.J., Vaidya, A., Long, C. and Sharma, S. 2000b. Antibodies against ribosomal phosphoprotein P0 of *Plasmodium falciparum* protect mice against challenge with *Plasmodium yoelii*. Infect. Immun. 68:4312-4318.

Fujii, K., Takabatake, N., Boonchit, S., Hirata, H., Yokoyama, N. and Igarahsi, I. 2002. Immunoscreening of *Babesia rodhaini* genes encoding immunogenic proteins from cDNA ecpression phage library. J. Protozool. Res. 12:34-42.

Fukasawa, M., Shimizu, Y., Shikata, K., Nakata, M., Sakakibara, R., Yamamoto, N., Hatanaka, M. and Mizuochi, T. 1998. Liposome oligomannose-coated with neoglycolipid, a new candidate for a safe adjuvant for induction of CD8+ cytotoxic T lymphocytes. FEBS Lett. 441:353-356.

Goswami, A., Chatterjee, S. and Sharma, S. 1996. Cloning of a ribosomal phosphoprotein P0 gene homologue from *Plasmodium falciparum*. Mol. Biochem. Parasitol. 82:117-120.

Goswami, A., Singh, S., Redkar, V.D. and Sharma, S. 1997. Characterization of P0, a ribosomal phosphoprotein of *Plasmodium falciparum*. Antibody against amino-terminal domain inhibits parasite growth. J. Biol. Chem. 272:12138-12143.

Homer, M.J., Aguilar-Delfin, I., Telford, S.R.3rd, Krause, P.J. and Persing, D.H. 2000. Babesiosis. Clin. Microbiol. Rev. 13:451-469.

Igarashi, I., Suzuki, R., Waki, S., Tagawa, Y., Seng, S., Tum, S., Omata, Y., Saito, A., Nagasawa, H., Iwakura, Y., Suzuki, N., Mikami, T. and Toyoda, Y. 1999. Roles of CD4(+) T cells and gamma interferon in protective immunity against *Babesia microti* infection in mice. Infect. Immun. 67:4143-4148.

Ikehara, Y. and Kojima, N. 2007. Development of a novel oligomannose-coated liposome-based anticancer drug-delivery system for intraperitoneal cancer. Curr. Opin. Mol. Ther. 9:53-61.

Iseki, H., Takabatake, N., Ota, N., Ishigame, T., Yokoyama, N. and Igarashi, I. 2008. *Babesia*: the protective effects of killed Propionibacterium acnes on the infections of two rodent *Babesia* parasites in mice. Exp. Parasitol. 118:543-548.

Kamiya, S., Takabatake, N., Fujii, K., Okamura, M., Iseki, H., Yokoyama, N. and Igarahsi, I. 2005. Protective immunity of blood plasma fraction containing *Babesia rodhaini* exoantigens in mice. J. Protozool. Res. 15:29-40.

Kojima, N., Biao, L., Nakayama, T., Ishii, M., Ikehara, Y. and Tsujimura, K. 2008. Oligomannose-coated liposomes as a therapeutic antigen-delivery and an adjuvant vehicle for induction of *in vivo* tumor immunity. J. Control. Release. 129:26-32.

Kuboki, N., Tiwananthagorn, W., Takagi, H., Nakayama, T., Xuan, X., Inoue, N., Igarashi, I., Tsujimura, K., Ikehara, Y., Kojima, N. and Yokoyama, N. 2007. Preliminary evaluation of oligomannose-coated liposome vaccines against lethal protozoan infections in mice. J. Protozool. Res. 17:9-15.

Mizuochi, T., Loveless, R.W., Lawson, A.M., Chai, W., Lachmann, P.J., Childs, R.A., Thiel, S. and Feizi, T. 1989. A library of oligosaccharide probes (neoglycolipids) from N-glycosylated proteins reveals that conglutinin binds to certain complex-type as well as high mannose-type oligosaccharide chains. J. Biol. Chem. 264:13834-13839.

Mosmann, T.R. and Moore, K.W. 1991. The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol. Today. 12:A49-53.

Nishisaka, M., Yokoyama, N., Xuan, X., Inoue, N., Nagasawa, H., Fujisaki, K., Mikami, T. and Igarashi, I. 2001. Characterisation of the gene encoding a protective antigen from *Babesia microti* identified it as eta subunit of chaperonin containing T-complex protein 1. Int. J. Parasitol. 31:1673-1679.

Perriere, G. and Gouy, M. 1996. WWW-query: an on-line retrieval system for biological sequence banks. Biochimie. 78:364-369.

Qing, G., Ma, L.C., Khorchid, A., Swapna, G.V., Mal, T.K., Takayama, M.M., Xia, B., Phadtare, S., Ke, H., Acton, T., Montelione, G.T., Ikura, M. and Inouye, M. 2004. Cold-shock induced high-yield protein

production in *Escherichia coli*. Nat. Biotechnol. 22:877-882.

Reiner, S.L. 1994. Parasites and T helper cell development: some insights. Parasitol. Today. 10:485-488.

Rich, B.E. and Steitz, J.A. 1987. Human acidic ribosomal phosphoproteins P0, P1, and P2: analysis of cDNA clones, *in vitro* synthesis, and assembly. Mol. Cell. Biol. 7:4065-4074.

Santos, C. and Ballesta, J.P. 1994. Ribosomal protein P0, contrary to phosphoproteins P1 and P2, is required for ribosome activity and *Saccharomyces cerevisiae* viability. J. Biol. Chem. 269:15689-15696.

Sher, A. and Coffman, R.L. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu. Rev. Immunol. 10:385-409.

Shimada, T., Shikano, S., Hashiguchi, R., Matsuki, N. and Ono, K. 1996. Effects of depletion of T cell subpopulations on the course of infection and anti-parasite delayed type hypersensitivity response in mice infected with *Babesia microti* and *Babesia rodhaini*. J. Vet. Med. Sci. 58:343-347.

Shimizu, Y., Takagi, H., Nakayama, T., Yamakami, K., Tadakuma, T., Yokoyama, N. and Kojima, N. 2007. Intraperitoneal immunization with oligomannose-coated liposome-entrapped soluble leishmanial antigen induces antigen-specific T-helper type immune response in BALB/c mice through uptake by peritoneal macrophages. Parasite. Immunol. 29:229-239.

Shimizu, Y., Yamakami, K., Gomi, T., Nakata, M., Asanuma, H., Tadakuma, T. and Kojima, N. 2003. Protection against *Leishmania major* infection by oligomannose-coated liposomes. Bioorg. Med. Chem. 11:1191-1195.

Shkap, V., de Vos, A.J., Zweygarth, E. and Jongejan, F. 2007. Attenuated vaccines for tropical theileriosis, babesiosis and heartwater: the continuing necessity. Trends. Parasitol. 23:420-426.

Takagi, H., Furuya, N. and Kojima, N. 2007. Preferential production of IL-12 by peritoneal macrophages activated by liposomes prepared from neoglycolipids containing oligomannose residues. Cytokine 40:241-250.

Terkawi, M.A., Jia, H., Zhou, J., Lee, E.G., Igarashi, I., Fujisaki, K., Nishikawa, Y. and Xuan, X. 2007. *Babesia gibsoni* ribosomal phosphoprotein P0 induces cross-protective immunity against *B. microti* infection in mice. Vaccine 25:2027-2035.

Terkawi, M.A., Zhang, G., Jia, H., Aboge, G., Goo, Y.K., Nishikawa, Y., Yokoyama, N., Igarashi, I., Kawazu, S.I., Fujisaki, K. and Xuan, X. 2008. C3 contributes to the cross-protective immunity induced by *Babesia gibsoni* phosphoriboprotein P0 against a lethal *B. rodhaini* infection. Parasite. Immunol. 30:365-370.

Yokoyama, N., Bork, S., Nishisaka, M., Hirata, H., Matsuo, T., Inoue, N., Xuan, X., Suzuki, H., Sugimoto, C. and Igarashi, I. 2003. Roles of the Maltese cross form in the development of parasitemia and protection against *Babesia microti* infection in mice. Infect. Immun. 71:411-417.

Yokoyama, N., Okamura, M. and Igarashi, I. 2006. Erythrocyte invasion by *Babesia* parasites: current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. Vet. Parasitol. 138:22-32.