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Protective immunity of blood plasma fraction containing *Babesia rodhaini* exoantigens in mice

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ABSTRACT

Babesia spp. are major hemoprotozoan parasites in animals. Exoantigens, which are soluble antigens released by the parasites during their asexual development, have been successfully applied for the development of a vaccine against babesiosis. In this study, we examined the ability of exoantigens to induce protective immunity in mice against a rodent *Babesia* parasite, *Babesia rodhaini*. The BALB/c mice immunized with the blood plasma fraction of *B. rodhain*i-infected mice (*B. rodhaini* exoantigens) showed a significant decrease in the lethal rate when challenged with *B. rodhaini*. Western blot analysis with anti-*B. rodhaini* exoantigen immune serum specifically detected at least eleven proteins in the lysate of *B. rodhaini*-infected erythrocytes. Next, a cDNA expression phage library of *B. rodhaini* was immunoscreened with the anti-*B. rodhaini* exoantigen immune serum, and three positive cDNA clones were obtained. However, compared to control mice, the mice immunized with each of these recombinant proteins expressed in *E. coli* or insect cells did not show significantly prolonged survivals after the challenge infection. These findings indicated that *B. rodhain*i-derived exoantigens contain vaccine candidates inducible of protective immunity in mice, but further investigation is needed to identify the target antigens responsible for the protection.

INTRODUCTION

Babesiosis of demostic animals causes great economic losses in the livestock industry worldwide (Krause, 2002; Kuttler, 1988). *Babesia* parasites destroy host erythrocytes (RBC) and induce fever, anemia, and icterus in the infected animals (Irwin, 2002). Although several chemotherapeutic treatments are available for infected animals (Wijaya et al., 2000), vaccine prevention would be more preferable for the rational approach. Live vaccines have been used to control the disease; however they have serious disadvantages, such as the limited shelf-life, variable infectivity and safety, and the risk of contaminations with other pathogens (Lora, 1981; Montenegro-James et al., 1992). In contrast, immunogenicity through a non-living

babesial vaccine has been described in *Babesia bovis*, but the vaccination was reported to induce only partial protection in cattle (Mahoney et al., 1967). Additionally, the immunogenicities of separated fractions from *B. bovis*-infected RBC have been evaluated in the host (Goodger et al., 1992), but no promising results have been obtained (Brown, 2001).

In *B. bovis*, exoantigens, which are parasite-free soluble antigens released into the *in vitro* culture medium during their asexual development (James et al., 1981), have been reported to induce protective immunity in cattle and result in no mortality of vaccinated cattle against the challenge infection (Smith et al., 1981). This strategy has also been successfully applied for other *Babesia* species, such as *B. bigemina* (Montenegro-James et al., 1987), *B. canis* (Schetters et al., 1992, 2001), and *B. divergens* (Gorenflot et al., 1991; Precigout et al., 2004). Thus, babesial exoantigens are expected to contain potential candidates capable of inducing protective immunity against babesiosis. Therefore, the identification of the babesial exoantigens responsible for the protection has become important for the development of a new babesial vaccine.

B. rodhaini, which causes a lethal infection in mice, can be used in *in vivo* experiments as a useful model for the babesiosis of many other animals (Shimada et al., 1991). In this report, we examined the protective effect of *B. rodhaini*-derived exoantigens against a subsequent challenge infection with *B. rodhaini*. Furthermore, three kinds of *B. rodhaini* cDNA clones were obtained by an immunoscreening technique, and the protective effects of these recombinants were evaluated upon the challenge infection.

Materials and methods

Parasitea and mice.

The Australian strain of *B. rodhaini* (Shimada et al.1991) was maintained by blood passage in mice (Nishisaka et al., 2001). Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). All of the mice were between 6 and 8 weeks old at the beginning of the *in vivo* experiments.

Protective effect of B. rodhaini exoantigens in mice.

B. rodhaini-infected erythrocytes (IRBC) of approximately 1 x 10^8 were intraperitoneally (i.p.) inoculated into uninfected mice. Blood was collected with a heparinized syringe from the infected mice on day 3 after the inoculation, when the mice showed a developing phase of parasitemia (approximately 30%) but did not induce any detectable antibodies against *B. rodhaini* (data not shown). Plasma fraction was isolated from the blood by low centrifugation at 350 x g for 10 min at 4℃ and then clarified by high centrifugation at 15,000 x g for 10 min at 4℃ to use as *B. rodhaini* exoantigens. As the control, a normal blood plasma fraction was also collected from uninfected mice in the same manner.

Mice were divided into three groups, designated as A (n=8), B (n=9), and C (n=8), for the *in vivo* vaccine trial. Group A or B received four i.p. immunizations with the *B. rodhaini* exoantigens or normal blood plasma fraction, respectively, at 14-day intervals. In detail, a 100 μl aliquot of each antigenic fraction was mixed with the same amount of a complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) and given to each mouse of the corresponding group as the first immunization. For the subsequent immunizations, the fractions were mixed with an incomplete Freund's adjuvant (Difco Laboratories) and given as described above. The mice in group C did not receive any treatments. On day 10 after the final immunization, all of the

mice were i.p. challenged with 1 x 10^7 *B. rodhaini*-IRBC. After the challenge, Giemsa-stained thin blood smears from the tail veins were prepared daily (Nishisaka et al., 2001), and the parasitemia and survival rate were monitored for a total of 15 days or until the death of the mice. All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

Indirect immunoflourescent antibody test (IFAT)

B. rodhaini-IRBC were collected from the infected mice (approximately 10% parasitemia) and washed three times with phosphate-buffered saline (PBS). Thin smears were prepared on glass slides, fixed with absolute methanol for 1 min at -20℃, and subjected to IFAT with the anti-*B. rodhaini* exoantigen immune serum obtained as described below. Alexa Flour 488 goat anti-mouse immunoglobulin G (IgG) (Molecular Probes, Inc. Eugene, Oregon, USA) and propidium iodide (Molecular Probes, Inc.) were applied as described previously (Yokoyama *et al*., 2003). Slides were finally analyzed using confocal laser scanning microscopy (TCS NT; Leica, Heidelberg, Germany).

Western blot analysis

B. rodhaini-IRBC or uninfected RBC were denatured by mixing with an equal amount of a 2×sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 10% 2-mercaptoethanol, 4% SDS, and 0.05 % bromophenol-blue) and subjected to electrophoresis on a 10% or 15% polyaclylamide gel. Western blot analysis was performed with the anti-*B. rodhaini* exoantigen immune serum as described previously (Yokoyama *et al*., 2003). Positive reactions were then visualized with a Western Lighting Chemiluminescence Regent Plus (Perkin Elmer Lifesciences, Boston, MA, USA) with a Versa Doc Imaging System (Bio-Rad, Hercules, CA, USA).

Immunoscreening and nucleotide sequencing

The *B. rodhaini*-cDNA *expression* phage library (Fujii *et al*., 2002) was screened with the anti-*B. rodhaini* exoantigen immune serum, and the nucleotide sequences of the isolated cDNAs were determined by the methods descrived previously (Nishisaka *et al*., 2001).

Expressions of recombinant proteins in Escherichia coli (E. coli) and insects cells

Insert cDNAs of Br-2, Br-4, and Br-10 genes were amplified using PCR with appropriate primers designed at the extreme 5'- and 3'-ends of the genes with appropriate restriction enzyme cleavage sites. The amplified DNA fragments were digested with the corresponding enzymes and ligated into the cloning site of an *E. coli* expression plasmid vector pGEMEX-2 (Promega, Madison, WI, USA) or the baculovirus transfer vector $pF_{AST}B_{AC}$ DUAL (GIBCO BRL, Rockville, MD, USA). The resultant plasmids were designated as pGEMEX-2/Br-2, pGEMEX-2/Br-4, and pGEMEX-2/Br-10 for the *E. coli* expression and $pF_{AST}B_{AC}$ DUAL/Br-2, $pF_{AST}B_{AC}$ DUAL/Br-4 and $pF_{AST}B_{AC}$ DUAL/Br-10 for the baculoviral expression, respectively. In *E. coli* JM109 (DE3), the recombinant products were expressed and purified as insoluble proteins fused with T7 gene 10 (gene10-Br-2, gene10-Br-4, and gene10-Br-10, respectively) according to the manufacturer's instructions (Promega). These insoluble proteins were used for the immunization of mice to produce the specific antibodies against the Br-2, Br-4, and Br-10 proteins. On the other hand, for the generation of recombinant baculoviruses, each of the baculovirus transfer vectors was transformed into *E.*

coli DH10Bac (Life Technologies Inc., Rockville, MD, USA). The resultant transposed bacmids containing the Br-2, Br-4, and Br-10 genes were selected and then transfected into Sf9 insect cells as described previously (Yokoyama *et al*., 1999). Recombinant baculoviruses were obtained from the supernatant of transfected cells and designated as AcBr-2, AcBr-4, and AcBr-10, respectively. AcFBD was generated with the pFast Bac Dual vector without any insertions to use as a control virus for the present study. The expression of recombinant proteins was performed in High Five (HF) insect cells as described previously (Yokoyama *et al*., 1999)**.**

Production of the mouse immune sera against B. rodhaini exoantigens and recombinant proteins

Five mice were immunized four times with the *B. rodhaini* exoantigens as described above. On day 10 after the final immunization, the sera were collected from the mice and used as anti-*B. rodhaini* exoantigen immune serum. On the other hand, to obtain the specific immune sera against the Br-2, Br-4, and Br-10 proteins, 100 μg of the recombinant gene10-fused proteins in a Titer Max GOLD (Titer Max USA Inc., GA, USA) was i.p. injected three times to BALB/c mice at intervals of 2 weeks. Sera were collected from the immunized mice on day 10 after the last immunization.

Protect effects of the recombinant proteins in mice

Insoluble gene10-fused proteins or lysates of the baculovirus-infected HF cells were used for immunizing five mice in each group with Freund's adjuvants. In detail, five BALB/c mice were i.p. immunized three times with 0.2 mg of the indicated antigens in adjuvants at 2-week intervals, where the primary immunization was performed with a Freund's complete adjuvant and the subsequent immunizations with a Freund's incomplete adjuvant as described previously (Yokoyama *et al*., 2003). Mice were then challenged with 1×10^7 IRBC at 10 days after the last immunization, and the survival rates were monitored as described above. The control mice received only PBS.

Statistical analyses

Survival rates of infected mice were plotted according to a Kaplan-Meier method and the statistical significance in the survival rate was analyzed among the groups by the generated Wilcoxon test, in where *p*-values of <0.05 were considered statistically significant.

RESULTS

Protective immunity of B. rodhaini exoantigens in mice

In order to examine protective immunity of *B. rodhaini* exoantigens, mice were immunized four times with the exoantigens (blood plasma fraction of *B. rodhaini*) and subsequently challenged with *B. rodhaini*. After the challenge infection, all of the non-immunized mice showed a typical development of high parasitemia and then died (group C; Figs.1C and D). On the other hand, the development of parasitemia and the lethal rate were clearly prevented and held down, respectively, in the exoantigen-immunized mice of group A (Figs. 1A and D). Although only two mice (Nos. 1 and 4) died on days 5 and 6, respectively, other mice survived the challenge infection. Among the six survivors of group A, the maximal level of parasitemia was 25.2 % on day 7 in a mouse (No. 5), and the parasite completely disappeared after the 11th day. In the mice of group B immunized with a normal blood plasma fraction, a typical development of high parasitemia

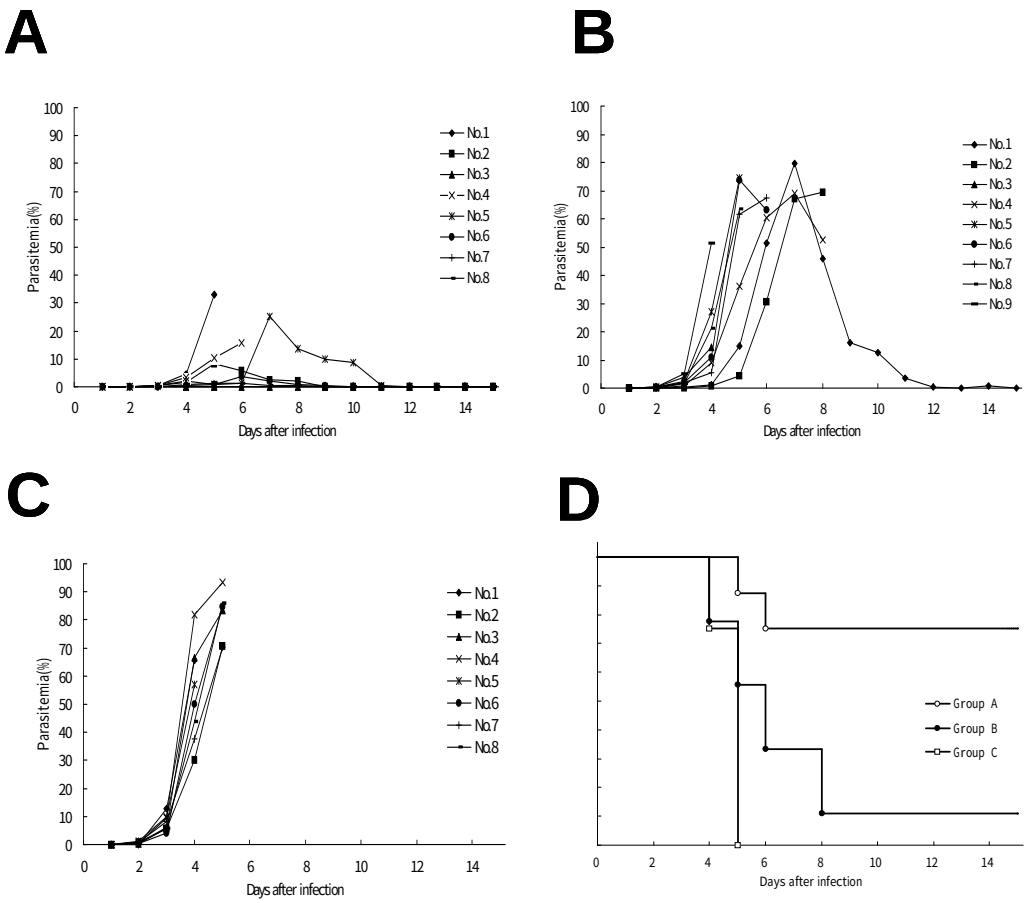

Fig. 1. Parasitemia and survival rates in mice challenged with *B. rodhaini* after the immunizations. Mice in groups A or B were immunized four times with the *B. rodhaini* exoantigen (**A**) or the normal mouse blood plasma fraction (**B**), respectively. No immunization was carried out in group C (**C**). Panel D shows the survival rates for each group that received *B. rodhaini* exoantigens (group A), normal mouse blood plasma fraction (group B), or non-immunogen (group C).

was observed, as seen in group C, but only one mouse (No. 1) survived (Figs.1B and D). The parasitemia reached 79.8 %, but the parasite disappeared on day 15. Except for this mouse, all mice died between days 4 and 8, at the peak of parasitemia (24.5-74.7%). Consequently, as shown in Figure 1D, both groups B and C showed extremely high lethal rates on day 15, while 75% of the mice in group A remained survived the challenge infection, indicating a significant difference ($p<0.05$) between the group A and others.

Immunological analyses with anti-B. rodhaini exoantigen immune serum

In order to investigate the reactive antigens with the mouse immune serum against *B. rodhaini* exoantigens, IFAT and Western blot analysis were carried out. In IFAT with the anti-*B. rodhaini* exoantigen immune serum, positive reactions were often observed mainly with extracellular merozoites and intracellularly multi-dividing parasites but also weakly around the surface of *B. rodhaini*-IRBC; however, some of single intra-erythrocytic parasites did not react or weekly reacted (Figs. 2A and B). IFAT with normal mouse serum did not show any significant fluorescence on the parasites (data not shown). In Western blot analysis with the anti-exoantigen immune serum (Fig. 2C), at least eleven proteins were specifically detected in the lysate of *B. rodhaini*-IRBC (lane 2), as compared with non-infected RBC lysate (lane 1). The sizes of these antigens were determined to be as follows: 140, 99, 97, 80, 66, 58, 40, 37, 35, 33, and 20 kDa.

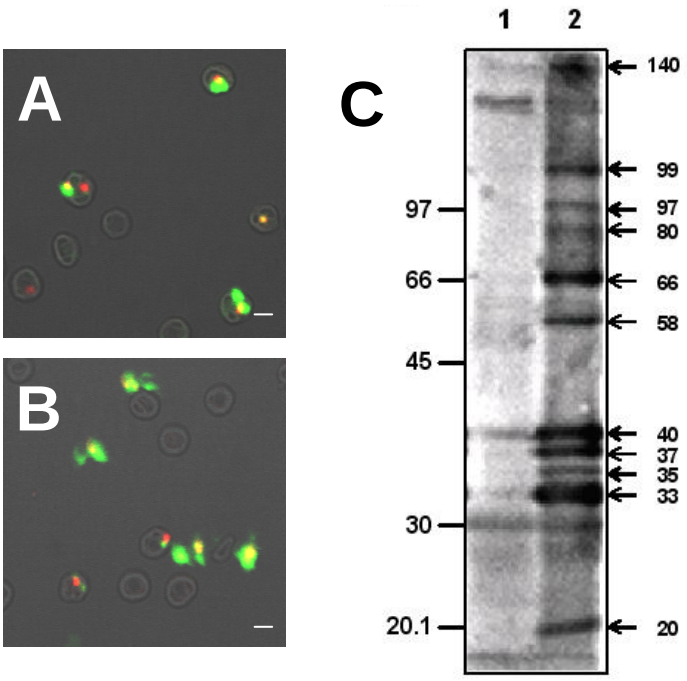

Fig. 2. Localization of exoantigens observed by IFAT using a conforcal laser scanning microscope. Methanol-fixed smears of *B. rodhaini*-IRBC were incubated with anti-*B. rodhaini* exoantigen immune serum (panels **A** and **B**). The antibody-antigen reaction (green) and nucleus (red) were visualized with the Alexa Flour 488-conjugated secondary antibody and propidium iodide, respectively. Bars, 5 μm. Panel **C** shows the result of Western blot analyses. The antigens prepared from *B. rodhaini*-infected (lane 2) or non-infected (lane 1) RBC were reacted with anti-*B. rodhaini* exoantigen immune serum and visualized with an ECL kit. The positions of the molecular mass standards are shown on the left of the panel (in kilodaltons). The arrows indicate the positions of the positive bands with their molecular weights.

The reactions of eight bands (140, 99, 66, 58, 40, 37, 33, and 20 kDa) were specifically more intense and detectable than those of the other three bands.

Immunoscreening of B. rodhaini-cDNA expression phage library and characterization of the gene products

The *B. rodhaini*-cDNA expression phage library was screened with the anti-*B. rodhaini* exoantigen immune serum. Twenty-one cDNA clones were obtained by immunoscreening and summarized into three groups by aligning the putative amino acid sequences of the identified open reading frames. Table 1 shows the list of positive cDNA clones containing the longest inserts among identical members of one group. The GenBank accession numbers of Br-2, Br-4, and Br-10 genes are AB103579, AB103581, and AB103587, respectively. For the detection of the native *B. rodhaini* proteins of Br-2, Br-4, and Br-10, these specific immune sera were prepared in mice against gene10-Br-2, gene10-Br-4, and gene10-Br-10 proteins (136, 60, and 100 kDa, respectively) (data not shown). Specific antibodies to the Br-4 and Br-10 proteins clearly reacted with the cytoplasmic area of *B. rodhaini* parasites (Figs. 3Bb and Cb), while the specific antibody to Br-2 protein strongly reacted to a whole region of *B. rodhaini*-IRBC (Fig. 3Ab). Control immune serum against the gene 10 protein did not show any reactions with the parasites and IRBC (data not shown). In Western blotting analyses, the specific antibody to the Br-2 protein recognized 83 and 75 kDa of the proteins in the lysate of *B. rodhaini*-IRBC (Fig. 3Aa). On the other hand, the bands of 32 and 28 kDa were detected with the specific antibody to the Br-4 protein (Fig. 3Ba) and a single band of 68 kDa with the specific antibody to the Br-10 protein (Fig. 3Ca).

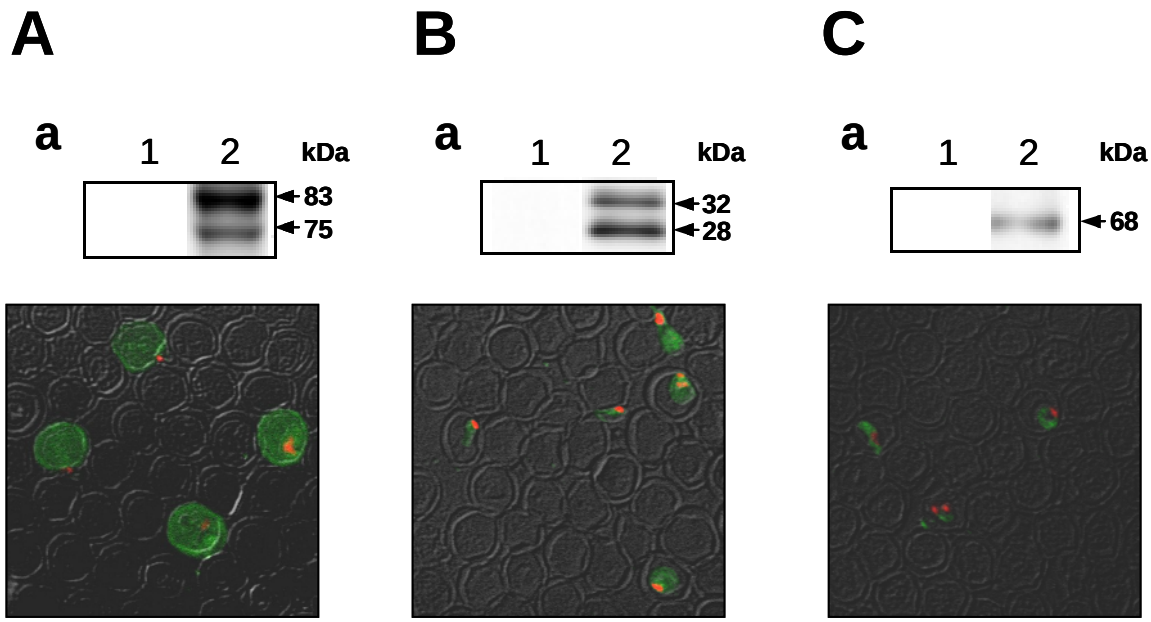

Fig. 3. Reactivities of anti-recombinant antigen-specific antibodies with *B. rodhaini*-IRBC. Panels **A-a**, **B-a**, and **C-a** show the results of Western blot analyses. The lysate of non-infected (lane1) or *B. rodhaini*-infected (lane 2) RBC was reacted with the anti-gene10-Br-2 (A-a) or anti-gene10-Br-4 (B-a), or with anti-gene10-Br-10 specific antibodies (C-a). The arrows indicate the positions of positive bands with their molecular weights (in kilodaltons). Panels **A-b**, **B-b**, and **C-b** show the results of IFAT. Methanol-fixed smears of *B. rodhaini* IRBC were incubated with the anti-gene10-Br-2 (A-b) or anti-gene10-Br-4 (B-b) or with anti-gene10-Br-10 specific antibodies (C-b). Bars=5 μm.

Immunization trials of recombinant antigens

To investigate whether these three antigens would become vaccine candidates for rodent babesiosis, E. *coli*-expressed or baculovirus-expressed recombinant antigens were immunized to mice three times, and the protective effects against *B. rodhaini* lethal infection were investigated. Mice immunized with gene10-Br-2, gene10-Br-4, and gene10-Br-10 expressed in *E. coli* did not survive the infection (Fig. 4A). All mice died within 4-9 days of the infection, and there was no significant difference in the survival rates among the groups. On the other hand, in another experiment with baculovirus-expressed antigens, although the mice immunized with recombinant Br-4, Br-2, and Br-10 antigens in the infected HF cell lysates showed 60, 25, and 20% survival rates, respectively, the AcFBD group also showed a high survival rate of 40%. Furthermore, no statistical difference was observed between the recombinant antigen-immunized and control baculovirus groups (Fig. 4B). In both *in vivo* experiments, all mice in the control group that had received only PBS before the challenge infection showed a typical development of high parasitemia (data not shown) and died within 4-7days (Fig. 4).

DISCUSSION

In the present study, *B. rodhaini*-derived exoantigens were found to induce significantly protective immunity against the lethal infection of *B. rodhaini* as described in other *Babesia* parasites (Smith *et al*., 1981; Montenegro-James *et al*., 1987; Schetters *et al*., 1992, 2001; Gorenflot *et al*., 1991; Precigout *et al*., 2004). Since IFAT with the anti-*B. rodhaini* exoantigen immune serum showed positive reactions mainly with *B. rodhaini* extracellular merozoites and intracellularly divided parasites, the crude exoantigens possibly

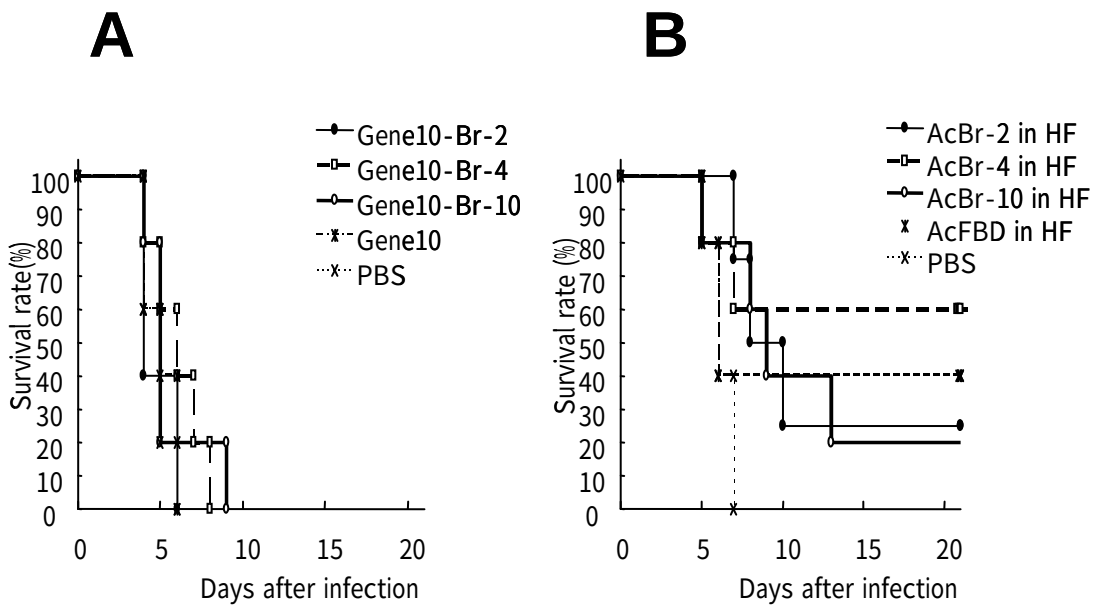

Fig. 4. Survival rates for mice challenged with *B. rodhaini* after the immunizations. Each group was immunized with gene10-Br-2, gene10-Br-4, gene10-Br-10, or only gene 10 in panel **A** or the lysate of the recombinant AcBr-2-, AcBr-4-, AcBr-10-, or AcFBD-infected HF cells in panel **B**. Control mice received PBS (**A** and **B**). AcBr-2, AcBr-4, and AcBr-10 expressed Br-2 of 84 kDa, Br-4 of 33 and 28 kDa, and Br-10 of 70 kDa in the infected HF cells, respectively (data not shown).

consist of functional antigens involved in the erythrocyte invasion. This finding suggests that some of the humoral antibodies raised against the exoantigens, which recognized at least eleven antigens in the lysate of IRBC, might prevent the parasites from erythrocyte infection in the immunized mice. However, it remains speculative yet, and further study will be required to understand the effect.

Next, we focused our efforts on a subset of *B. rodhaini* genes (Br-2, Br-4, and Br-10 genes) obtained by immunoscreening with the anti-*B. rodhaini* exoantigen immune serum. The Br-2 gene has a homologous sequence to *Plasmodium falciparum* asparagines- and aspartate-rich protein (AARP)-2 (Barale *et al.*, 1997) and contains asparagine- and aspartate-rich motifs in the amino acid sequence (data not shown). Since PfAARP-2, as well as a number of other asparagine-rich proteins, has been proposed to associate with the IRBC membrane (Franze'n *et al.*, 1989, Barale *et al.*, 1997), the biological role of the native Br-2 of 75 and 83 kDa, which is also located in the cytoplasmic area of *B. rodhaini*-IRBC, is presumed to be related with the modification of the erythrocyte cytoskeleton. The native Br-4 of 28 and 32 kDa has been regarded as a family of *B. rodhaini* surface membrane protein p26 (Snary and Smith, 1986, 1988). Br-4 and p26 proteins are close to each other, but there are several differences in their amino acid sequences (data not shown). The mechanism by which epitope variations occur between the Br-4 and p26 proteins in *B. rodhaini* might be attributed to an extensive polymorphism, presumably arising by genetic mutation (O'Donnell *et al.*, 2000; Fisher *et al.*, 2001). The Br-4 also showed homologies to the *P. falciparum* merozoite surface protein (MSP)-3 (Kumar *et al.*, 2002) and the *P. vivax* reticulocyte binding protein (PvRBP)-1 (Galinski and

Table 1 List of immune reactive cDNA clone with anti-*B. rodhaini*-exoantigens immune serum

Name	Sequence length (bp)	Homologous Protein*	Origin	Acc. No.	Expect
Br-2	2257	BMN1-8	*B. microti*	AF206251-1	7E-14
		AARP-2	*P. falciparum*	Y08924-1	0.062
Br-4	2116	p26	*B. rodhaini*	M19145-1	E-122
		p25	*B. rodhaini*	M19145-2	1E-50
		MSP-3	*P. falciparum*	AE014834-39	0.005
		PvRBP-1	*P. vivax*	AY501889-1	0.006
Br-10	2113	HSP70	*B. microti*	U53448-1	0
		HSP70	*Toxoplasma gondii*	U85649-1	0
		HSP70	*P. falciparum*	M19753-1	0

*Diverse proteins showing homologous amino acid sequences against the indicated proteins through a BLAST search to NCBI database.

Barnwell, 1996) based on parts of the amino acid sequence, suggesting a possible involvement in the erythrocyte invasion of Br-4. Br-10 has high homology to the family of the 70kDa heat shock protein (HSP70) (Erol *et al*., 1999; Heussler *et al*., 2001; Sharma, 1992) in the amino acid sequence. HSP70 genes have been reported from a number of apicomplexan parasites, including *B. bovis* (Ruef *et al*., 2000), *B. microti* (Erol *et al*., 1999), and *Plasmodium* spp. (Bianco *et al*.,1986). Due to the ability of HSP70 to regulate parasite actin polymerization, protozoan HSP70 is considered to play an important role in the host cell invasion (Tardieux *et al*., 1998).

In previous studies, immunizations with recombinant p26 and HSP70 antigens induced some degree of protective immunity in mice against *B. rodhaini* and *B. microti* infections, respectively (Igarashi *et al*., 2000; Erol *et al*., 1999). However, compared to the control mice, the mice immunized with Br-2, Br-4, and Br-10 recombinant antigens did not show any significantly prolonged survival rates after the *B. rodhaini* challenge infection. Further investigation would be required to identify the vaccine candidates capable of controlling the asexual growth of *B. rodhaini* in mice. Interestingly, the immunization of control baculovirus-infected insect cells showed partial protection against *B. rodhaini* infection. Recently, a live baculovirus was reported to stimulate an innate immune response by macrophage cells *in vitro* and *in vivo* (Abe *et al*., 2003). Considering the report, 40% of the surviving mice in the FBD group might be associated with the innate immune response. Due to its advantageous characteristics, such as low cytotoxicity in mammalian cells even at a high multiplicity of infection (McIntosh *et al*., 1980) and an inherent incapability to replicate in mammalian cells (Groner *et al*., 1984), the baculovirus could be useful as an adjuvant in the vaccine trial against babesiosis.

In the present study, *B. rodhaini* exoantigens showed protective immunity against the challenge infection

in mice. Additionally, we introduced three new babesial proteins but did not find novel molecules inducible for protective immunity from these proteins. Further investigation of other antigens, which were responsible for the observed protection by crude exoantigens and could be anti-babesial vaccine candidates, will be necessary.

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