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IMMUNOSCREENING OF *BABESIA RODHAINI* GENES ENCODING IMMUNOGENIC PROTEINS FROM THE CDNA EXPRESSION PHAGE LIBRARY

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Note: Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL, and DDBJ databases under the accession numbers.

ABSTRACT

Babesia parasites are major hemoprotozoa in a variety of animals, and the infections cause great economic losses in the livestock or companion animal industry worldwide. In this study, using anti-*B. rodhaini* immune serum, we examined the immunogenic proteins of *B. rodhaini*, which is a rodent species of *Babesia* and has been used as an experimental animal model for other babesioses. Immunological analyses with the immune serum showed wide localization in the parasite body as well as around the surface of infected red blood cells and the existence of at least 15 antigens. In addition, 10 groups of immunoreactive cDNA clones were obtained from a *B. rodhaini*-cDNA expression phage library by the immunoscreening technique with the immune serum. It is hoped that these results will supply useful materials for the development of babesial subunit vaccines.

Key words: *Babesia rodhaini*; Babesiosis; Immunoscreening; cDNA expression library.

INTRODUCTION

Babesia parasites transmitted by tick vectors are major hemoprotozoa in a variety of animals (Homer et al., 2000). The babesial infections cause great economic losses in the livestock or companion animal industry worldwide because they induce serious fever, anemia, and icterus in the infected animals and the infection often results in death (Kuttler, 1988). A live vaccine has been used for the control of babesiosis; however, this vaccine has serious disadvantages, such as limited shelf life, variable infectivity and safety, and risk via contaminations with other pathogens (Homer et al., 2000). In contrast, the immunogenicity of a non-living babesial vaccine has been reported, but this vaccine offers only partial protection to animals (Homer et al., 2000). Recently, subunit vaccines, as recombinant antigens derived from cloned cDNA, have been the focus of study for the development of preventive measures against protozoan infection of veterinary importance

(Jenkins, 2001). Particularly, the protozoan molecules involved in the invasion into host cells are of special interest (Preiser et al., 2000). These antigens have been shown to elicit sufficient immune response and afford satisfactory protection (Homer et al., 2000). At present, however, there is not enough information that could lead to the successful development of a subunit vaccine for babesioses.

Babesia rodhaini causes a lethal infection in mice (Igarashi et al., 1993). Therefore, we can use this parasite as an useful animal model for other babesioses. The acute infection can be cured by chemotherapy, and the recovered mice then become resistant to re-infection (Shimada et al., 1991). In the present study, we prepared anti-*B. rodhaini* immune serum in mice and determined the immunological characterization by an indirect immunofluorescent antibody test and Western blot analysis. Furthermore, we constructed a *B. rodhaini*-cDNA expression phage library and succeeded in obtaining 10 groups of 18 positive cDNA clones from the phage library by the immunoscreening technique with the immune serum. From the determined nucleotide sequences of cDNA inserts, we discussed the prospected roles of each gene product and its possible utility for the development of a vaccine against babesioses.

MATERIALS AND METHODS

Parasites and mice

An Australian strain of *B. rodhaini* was maintained by blood passage in BALB/c mice (Shimada et al., 1991). Infected red blood cells (IRBC) were collected from the infected mice with a heparinized syringe and diluted with phosphate-buffered saline (PBS) to a final concentration of 5×10^7 parasites/ml. Mice were intraperitoneally (i.p.) injected with 0.2 ml of the diluted IRBC, and the parasitemia was daily monitored in a thin blood smear stained with Gimsa solution. Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). All of the mice were between 6 and 8 weeks old at the starting time of the *in vivo* experiments.

Preparation of mouse anti-B. rodhaini immune serum

Five mice were i.p. inoculated with 1×10^4 infected RBC of *B. rodhaini*, followed by daily i.p. treatment with 25 mg/kg Ganaseg (diminazene diaceturate; Novartis Animal Health, Basel, Switzerland) over the course of 1 week, according to the previously described method (Shimada et al., 1991). At 4 weeks after the final treatment, the mice were inoculated again with *B. rodhaini* and subsequently treated with Ganaseg by the same method as above. At 4 weeks after the second inoculation, the mice were further inoculated with *B. rodhaini*, and the sera were collected from the surviving mice at 4 weeks after the final inoculation.

Indirect immunofluorescent antibody test (IFAT)

B. rodhaini-infected RBC (approximately 30% parasitemia) were collected from the infected mice and washed three times with cold PBS. Thin smears were prepared on slide glasses, dried, and then fixed with absolute methanol for 5 min at –20°C. The anti-*B. rodhaini* immune serum was applied as a primary

antibody on the fixed smear and incubated for 30 min at 37°C. After three washes with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobin G (IgG) (H+L) (Southern Biotechnology Associates Inc., Birmingham, Alabama, USA) was subsequently applied as a secondary antibody and incubated for 30 min at 37°C. After three washes with PBS, the smear was incubated with 25 μg of propidium iodide (PI) (Molecular probes, Eugene, Oregon, USA) and 50 μg of RNase A (Roche, Basel, Switzerland) per ml for 10 min at 37°C (Yokoyama et al., 2002, 2003). After three washes with PBS, the slides were mounted in 1% propyl gallate (Wako, Osaka, Japan) and 50% glycerol in PBS with cover slips and then observed by confocal laser scanning microscopy (TCS NT; Leica, Heidelberg, Germany).

Western blot analysis

B. rodhaini-infected or non-infected RBC were mixed with an equal amount of a 2 x SDS sample buffer (125 mM Tris-HCl (pH 6.8), 20% glycerol, 10% 2-mercaptoethanol, 4% SDS, and 0.05% bromophenol-blue) and heated at 100°C for 5 min. Ten microliters of the sample was loaded on a 10% polyacrylamide gel. The separated proteins were blotted onto a polyvinylidene difluoride membrane (Millipore, Watertown, Massachusetts, USA). After immersing in a blocking buffer (3% skim milk and 0.05% Tween-20 in PBS) for 2 h at room temperature (RT), the membrane was incubated with anti-*B. rodhaini* immune serum for 60 min at RT and subsequently with peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, USA) for 60 min at RT. Positive reactions were then visualized with a Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer Lifesciences, Boston, Massachusetts, USA).

Construction of a B. rodhaini-cDNA expression phage library

Total RNA was extracted from *B. rodhaini*-infected RBC with acid guanidinium thiocyanate phenol chloroform, and the polyadenylated mRNA was selected from the total RNA via an Oligotex dT 30 latex bead (Takara, Shiga, Japan). The double-strand cDNA with an *Eco*R I or *Xho* I linker at both terminal sites was synthesized from the mRNA using a Zap-cDNA synthesis kit (Stratagene, La Jolla, California, USA) and ligated to the *Eco*R I and *Xho* I sites of a λZap II phage gene expression vector (Strategene) as described previously (Nishisaka et al., 2001). The ligation mixture was packaged with a Gigapack III packaging system (Strategene).

Immunoscreening and nucleotide sequencing

The *B. rodhaini*-cDNA expression phage library was screened with the anti-*B. rodhaini* immune serum according to a previous report (Nishisaka et al., 2001). In brief, packed phages were grown on NZY agar and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, New Hampshire, USA) with an isopropyl-β-D-thiogalactopyranoside (Takara, Shiga, Japan). The membrane containing phage plaques was incubated with the anti-*B. rodhaini* immune serum for 60 min at RT and subsequently with an alkaline phosphatase-conjugated goat anti-mouse IgG (Strategene) and then visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Wako, Osaka, Japan). The positive phages were plaque-purified to

obtain a 100% clonal population. The cDNA insert in the λ phage was introduced into a pBluescript SK(+) vector (Strategene) by the *in vivo* excision capability of λ Zap II.

The nucleotide sequences of the positive cDNA inserts were determined using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) with an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Open reading frame (ORF) analysis and protein homology searches were performed using a Mac Vector program (Oxford Molecular Ltd., Oxford, UK) and the National Center for Biotechnology Information database, respectively.

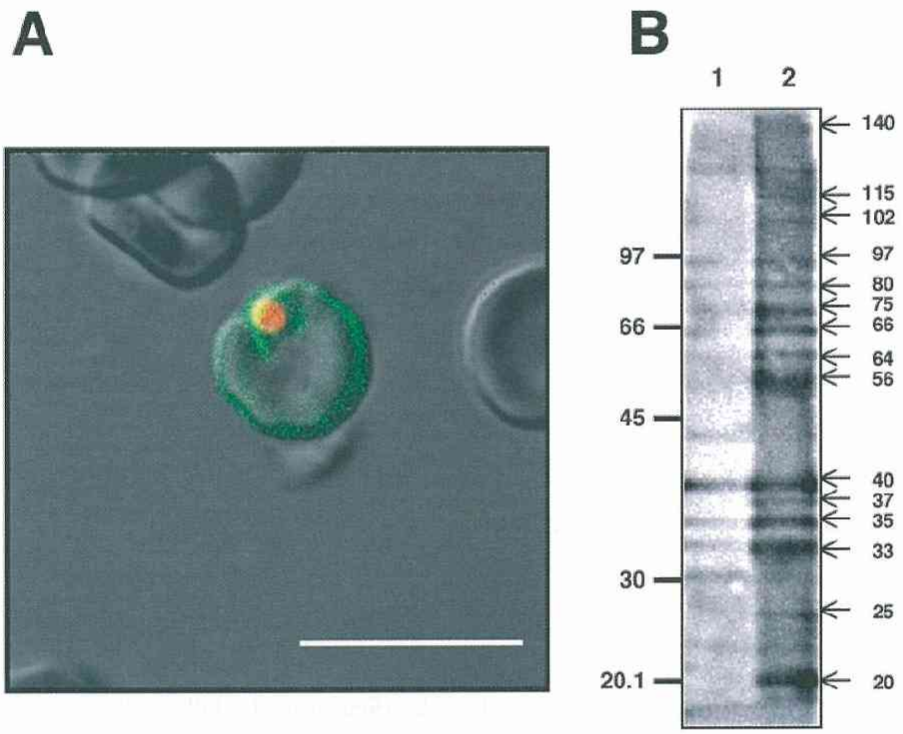

Fig. 1. A: IFAT using a confocal laser scanning microscope. Methanol-fixed smears of the *B. rodhaini*-infected RBC were incubated with anti-*B. rodhaini*-immune mouse serum. The antibody-antigen reaction (green) and nucleus (red) were visualized with the FITC-conjugated secondary antibody and PI staining, respectively. Bar=5 μm. **B**: Western blot analysis. The antigens prepared from non-infected RBC (lane **1**) and *B. rodhaini*-infected RBC (lane 2) were reacted with the anti-*B. rodhaini*-immune mouse serum. The position of the molecular mass standards is shown on the left of each panel (in kilodaltons). The molecular masses of each protein are indicated with arrows and the numerals.

RESULTS AND DISCUSSION

In order to investigate the reactivity with the prepared anti-*B. rodhaini* immune serum, IFAT and Western blot analysis were carried out. In IFAT, positive reactions with the immune serum were observed not only in all of the intraerythrocytic parasites but also around the surface of infected RBC (Fig. 1A). Additionally, extracellular merozoites were also detectable with the immune serum (data not shown). On the other hand, fluorescence was not found in normal RBC. These findings indicated that the immunoreactive

antigens were widely distributed in the entire parasite and were also located on the membrane and membrabe-skeleton of infected RBC. In Western blot analysis with the immune serum (Fig. 1B), at least 15 proteins were specifically detected in the *B. rodhaini*-infected RBC lysate (lane 2), as compared to the control result of the non-infected RBC lysate (lane 1). The sizes of these antigens were determined as follows: 140, 115, 102, 97, 80, 75, 66, 61, 56, 40, 37, 35, 33, 25, and 20 kDa. Seven of the bands (75, 66, 56, 40, 35, 33, and 20 kDa) were more clearly detectable than the others. These positive proteins were considered to be immunodominant antigens capable of inducing a humoral immune response in mice.

Next, a *B. rodhaini*-cDNA expression phage library was screened with the anti-*B. rodhaini* immune serum. As a result, 18 cDNA clones were successfully obtained by the immunoscreening technique. The nucleotide sequences of cDNA inserts were determined and grouped into 10 by aligning the putative amino acid sequences of the identified ORFs. Table 1 shows the list of representative cDNA clones containing the longest cDNA insert of each group. Homologous proteins to the putative amino acid sequences of determined ORFs are described below.

Table 1 List of the reactive cDNA clones with *B. rodhaini* immune serum

Group No.	Gene name	Accession No.	Length of putative ORF (amino acid)	Homologous protein	Origin	Accession No.	E value
1	Br-1	AB103578	521	BMN1-8	*B. microti*	AF206251	2.00E-11
				p26	*B. rodhaini*	B54497	5.00E-05
				MSP-1	*P. falciparum*	P04933	5.00E-01
2	Br-2	AB103579	684	BMN1-8	*B. microti*	AF206251	5.00E-14
				AARP-2	*P. falciparum*	Y08924	6.10E-02
				GLURP	*P. falciparum*	AF247634	2.00E+00
3	Br-3	AB103580	991	Py235	*P. yoelii yoelii*	AF323442	5.30E+00
4	Br-4	AB103581	273	p26	*B. rodhaini*	B54497	E-124
5	Br-5	AB103582	190	AARP-1	*P. falciparum*	Y08926	2.00E-04
				STARP	*P. falciparum*	AF209925	7.00E-03
6	Br-6	AB103583	347	MESA	*P. falciparum*	AF270648	3.00E-05
				Pf332	*P. falciparum*	AF202180	8.00E-04
				pypAg-1	*P. yoelii yoelii*	AF103869	1.00E-03
7	Br-7	AB103584	729	Py235	*P. yoelii yoelii*	U36927	5.00E-06
8	Br-8	AB103585	319	p25	*B. rodhaini*	A54497	E-164
9	Br-9	AB103586	525	PfCDPK	*P. falciparum*	X67288	E-153
				TgCDPK	*Toxoplasma gondii*	AF333958	E-144
				EtCDPK	*Eimeria tenella*	Z71757	E-141
10	Br-10	AB103587	645	HSP70	*B. microti*	U53448	0
				HSP70	*Toxoplasma gondii*	U85649	0
				HSP70	*P. falciparum*	M19753	0

Group No. 1: A protein of the BMN1-8 family was picked up from a *B. microti*-genomic expression phage library with *B. microti*-infected patient serum and considered to be a surface antigen with a large extracellular domain (Lodes et al., 2000). *Plasmodium falciparum* merozoite surface protein-1 (MSP-1) is a major candidate for the development of malaria vaccines (Kumar et al., 2002). Group No. 2: Group No. 2 has

limited homologies to *P. falciparum* asparagine/aspartate-rich protein (AARP) -2 (Barale et al., 1997a) and glutamate-rich protein (GLURP) (Oeuvray et al., 2000). GLURP, which is a peripheral membrane protein associated with the merozoite surface, was reported to induce a cytophilic antibody playing a protective role against *P. falcipalum* infection (Oeuvray et al., 2000). Group No. 3: *P. yoelii yoelii* 235-kDa rhoptry protein (Py235) has binding affinity to murine RBC and plays an important role in host cell selection (Khan et al., 2001). A reduction in the growth of the virulent *P. yoelii yoelii* strain has been reported in the immunized mice (Holder and Freeman, 1981). Group No. 4: Group No. 4 showed significantly high homology to *B. rodhaini* surface membrane protein, p26 (Snary and Smith, 1986, 1988). The p26 protein, which is believed to locate on the surface of merozoite, was shown to induce a degree of protective immunity in mice (Igarashi et al., 2000). Group No. 5: AARP-1, associated with the membrane of infected RBC, is considered to be a target of cytophilic antibodies to promote phagocytosis of the RBC (Barale et al., 1997b). *P. falciparum* sporozoite threonine- and asparagine-rich protein (STARP) is detectable on the surface of sporozoites and also expected to be a malaria vaccine target for the pre-erythrocytic stage (Fidock et al., 1997).

Group No. 6: The *P. falciparum* mature parasite-infected erythrocyte surface antigen (MESA) locates on the membrane skeleton of the infected RBC and plays a critical role in maintaining the mechanical integrity of the erythrocytic membrane (Coopel, 1992). The *P. falciparum* 332-kDa erythrocyte membrane-associated giant protein antigen (Pf332) is specifically expressed on the surface of infected RBC, and the specific antibodies inhibit parasite growth by interfering intraerythrocytic parasite development or schizont rupture (Ahlborg et al., 1996). Immunization with *P. yoelii yoelii* blood stage membrane protein Ag-1 (pypAg-1), which is expressed both in the cytoplasm and on the surface of infected RBC, was shown to induce a reduction in the level of parasitemia (Burns et al., 1999). Group No. 7: *P. falciparum* Py235 was described above. Group No. 8: Group No. 4 was regarded to be a *B. rodhaini* surface membrane protein, p25 (Snary and Smith, 1986, 1988). Group No. 9: All proteins that are homologous to the group No. 9 belong to a family of calmodulin-like protein kinases (CDPKs) that is involved in stress and hormone responses, germination, and passive membrane biogenesis (Dunn et al., 1996; Kappes et al., 1999; Kieschick et al., 2001). Group No. 10: Group No. 10 has significantly high homology to a 70-kDa heat-shock protein (HSP70) family of other protozoa (Erol et al., 1999; Heussler et al., 2001; Sharma, 1992). HSP70s are believed to assist the intracellular parasite in its adaptation to a new host environment during invasion and differentiation (Sharma, 1992) and also to inhibit the apoptotic program of the infected host cells (Heussler et al., 2001). Interestingly, immunization with the recombinant HSP70 induced partial protection in mice against the lethal challenge of *B. microti* (Erol et al., 1999).

In the present study, we succeeded in obtaining 10 groups of cDNA encoding *B. rodhaini* antigens. Based on the homology to other protozoa, almost all gene products were expected to be vaccine candidate antigens for *B. rodhaini* infection in mice. Analysis of their function would also provide clues to the understanding the asexually reproductive growth of *Babesia* parasite. Further studies, for example, the

determination of the cellular localization and protective effect of each gene product, will be necessary to understand its biological function and utility as a subunit vaccine. These trials would be applicable for the development of preventive measures against other animal babesioses.

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