INFECTION AND IMMUNITY, Jan. 2003, p. 411–417 Vol. 71, No. 1
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.1.411–417.2003

Roles of the Maltese Cross Form in the Development of Parasitemia and Protection against *Babesia microti* Infection in Mice

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Received 20 May 2002/Returned for modification 29 June 2002/Accepted 4 October 2002

***Babesia microti*, a hemoprotozoan parasite of rodents, is also important as a zoonotic agent of human babesiosis. The Maltese cross form, which consists of four masses in an erythrocyte, is characteristic of the developmental stage of *B. microti*. Monoclonal antibody (MAb) 2-1E, which specifically recognizes the Maltese cross form of *B. microti*, has been described previously. In the present study, we examined the roles of the Maltese cross form during the infectious course of *B. microti* in mice. The number of the Maltese cross form increased in the peripheral blood of infected mice prior to the peak of parasitemia. With confocal laser scanning microscopy, MAb 2-1E was found to be reactive with the ring form, with the parasites undergoing transformation to the Maltese cross form and subsequent division, and also with extracellular merozoites. Furthermore, the Maltese cross form-related antigen (MRA) gene was isolated from a *B. microti* cDNA library by immunoscreening with MAb 2-1E, and the nucleotide sequence was determined. Genomic analyses indicated that the MRA gene exists as a single-copy gene in *B. microti*. Immunization of mice with recombinant MRA induced significant protective immunity against *B. microti* infection. These findings indicate that the Maltese cross form plays important roles in both the development of parasitemia and the protective response against the infection.**

Babesiosis is a tick-transmitted protozoan infection caused by intraerythrocytic parasites of the genus *Babesia*, which infect a wide variety of wild and domestic animals and cause enormous economic losses throughout the world (16). *Babesia microti* is a rodent form of *Babesia* and is also known as a major etiologic agent of human babesiosis (9). Hundreds of human babesiosis cases caused by *B. microti* have been reported in the northeastern and upper midwestern areas of the United States (4, 7, 15, 18). Recently, *B. microti* infection has also been reported in Taiwan (25) and Japan (22). The disease manifestations of human babesiosis are caused by the asexually reproductive cycle of the merozoite in host erythrocytes, which induces subsequent lysis of the erythrocytes. The symptoms of human babesiosis are anemia, fever, emesis, and hematuria, among others, and the severity of the disease is believed to be associated with the level of parasitemia (2, 9, 21). Consequently, there is a very broad clinical spectrum, ranging from an apparently silent infection to a fulminant, malaria-like disease that occasionally results in death (9, 14).

B. microti has also been used as an experimental model to study immune mechanisms for babesiosis in many other animals. Mice infected with *B. microti* exhibit a transiently high parasitemia, but they subsequently recover from the acute infection. The mice that recover are protected against reinfection with *B. microti* (11, 12). Recently, it has been demonstrated that $CD4^+$ T cells play an essential role in the resolution of primary infection with *B. microti* (12, 26) and that gamma interferon produced by $CD4^+$ T cells is responsible for resolution of a primary or challenge infection with *B. microti* (11, 12).

Under a light microscope, ring and pear-shaped forms of *B. microti* are often seen in the infected erythrocytes. The Maltese cross form, however, which consists of four masses in an erythrocyte, is rarely seen, but it is often described as characteristic of *B. microti* (9, 13, 20), as well as *Babesia equi* (1, 24) (which was recently redescribed as *Theileria equi* [19]) and *Theileria parva* (5). However, the biology of these organisms and the role of the Maltese cross form are not fully understood. Monoclonal antibody (MAb) 2-1E, which specifically recognizes the Maltese cross form of *B. microti*, has been described previously (20). MAb 2-1E did not cross-react with any antigen of *Babesia rodhaini*, which is also a rodent *Babesia* parasite but does not develop the Maltese cross form in erythrocytes. In the present study, the roles of the Maltese cross form in the development of *B. microti* were studied. Furthermore, the Maltese cross form-related antigen (MRA) was identified by using MAb 2-1E, the gene encoding the MRA was isolated from a *B. microti* cDNA library, and the protective effect of the recombinant MRA gene product against a challenge infection with *B. microti* in mice was examined.

MATERIALS AND METHODS

Parasite and mice. *B. microti* strain Munich was maintained by blood passages with mice (20). Six-week-old female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan) and used for passage and experimental infections.

MAb. MAb 2-1E (20) was previously produced against the Maltese cross form of *B. microti*, and the supernatant of the cultured hybridoma was used for the analyses in this study.

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Indirect immunofluorescent antibody test (IFAT). *B. microti*-infected erythrocytes were collected from infected mice showing a developing phase of parasitemia (approximately 30%) and were washed three times with cold phosphate-buffered saline (PBS). Thin smears were prepared on slides and fixed with absolute methanol for 10 min at −20°C. MAb 2-1E or a mouse immune serum (see below) was applied as a primary antibody to the fixed erythrocytes and incubated for 30 min at 37°C. After three washes with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (ICN Pharmaceuticals, Irvine, Calif.) was used as a secondary antibody and incubated for 30 min at 37°C. After three washes with PBS, the slides were mounted in 50% glycerol with coverslips and used for confocal laser scanning microscopic observation (TCS NT; Leica, Heidelberg, Germany).

Immunoscreening and nucleotide sequencing of MRA cDNA clones. The *B. microti* cDNA library was screened with MAb 2-1E, and the complete nucleotide sequences of the isolated cDNAs were determined by the methods described previously (20). Sequence alignment and a homologous protein search were performed by using Mac Vector (AssemblyLIGN; Oxford Molecular Ltd., Oxford, United Kingdom) and the National Center for Biotechnology Information database, respectively.

Southern blot and PCR analyses. For the genomic analyses, total genomic DNA was extracted from *B. microti*-infected or noninfected blood by the standard method (23). In Southern blot analyses, 10 μg of *B. microti* genomic DNA was digested with a restriction endonuclease (see below) and separated on a 0.8% agarose gel. The separated DNAs were transferred to a nylon membrane (Hybond-N; Amersham-Buchler, Munich, Germany) and hybridized with a 5,510-bp DNA fragment. This fragment had been amplified from the cDNA clone containing an entire insert of the longest MRA gene by PCR with the standard primers, primers T3 and T7 (Stratagene, La Jolla, Calif.), and then labeled with alkaline phosphatase by using an AlkPhos Direct kit (Amersham Pharmacia Biotech, Piscataway, N.J.).

Two oligonucleotide primers (5′-AAACTCCGACTGTTGTTGG-3′ and 5′-TTAGCCGTGTTCAGAGACAG-3′) were designed by using the nucleotide sequences of MRA cDNA clones. Each PCR was performed in a 50-μl mixture (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin) containing 0.8 μg of the genomic DNA, 100 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 200 mM, and 2.5 U of *Taq* Gold DNA polymerase (Perkin-Elmer, Norwalk, Conn.). The mixture was heated for 10 min at 95°C to activate the *Taq* Gold DNA polymerase, and 30 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 5 min at 72°C for extension were performed. The amplified DNA samples were loaded on a 1% agarose gel.

Construction of recombinant baculoviruses. The cDNA clone containing the longest insert of the MRA gene was used to generate a recombinant donor plasmid, pMRA/FBD. The 5,361-bp DNA fragment, which artificially acquired an open reading frame of the MRA gene by connection of an ATG at the 5′ terminus, was amplified by PCR with the following primers: MRA-UP-*Pst*I (5′-ggctgcagatGGCACCAGGTGCTTTTCAAGAAATC-3′) and MRA-DN-*Hin*dIII (5′-ctcaagcTTCGTTGGTTCACAATTATGTTGATATC-3′) (lowercase letters indicate *Pst*I or *Hin*dI restriction site linkers; underlining indicates the start codon). The amplified DNA fragment was digested with *Pst*I and *Hin*dIII and then ligated between the *Pst*I and *Hin*dIII sites downstream of the polyhedrin promoter region of pFastBac-Dual (Life Technologies, Rockville, Md.). The technique used to generate a recombinant baculovirus has been described previously (27, 28). pMRA/FBD was transformed into DH10Bac-competent cells (Life Technologies), and the resultant transposed bacmid containing the MRA gene was selected and then transfected into Sf9 insect cells. A recombinant baculovirus, designated AcMRA, was collected from the supernatant of transfected cells. Another baculovirus, AcFB-D, was generated by using pFastBac-Dual without any insertions and was used as a control virus in the present study.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses. For purification of *B. microti* merozoites, *B. microti*-infected erythrocytes were treated with a 0.83% NH_4Cl solution for 10 min at 37°C and centrifuged at 2,000 × *g* for 10 min at 4°C (20). After three washes with cold PBS, the pellet containing merozoites was mixed with an equal amount of a 2× sodium dodecyl sulfate sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 10% 2-mercaptoethanol, 4% sodium dodecyl sulfate, 0.05% bromophenol blue). Baculovirus-infected Sf9 cells were also washed and mixed with the sample buffer. Each of the samples was heated at 100°C for 5 min. Ten microliters of each sample was loaded on a 10% polyacrylamide gel, and the gel was subjected to Western blot analyses.

For Western blot analyses, the separated proteins were blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.). The protein blots were incubated with MAb 2-1E for 60 min at 37°C and then with peroxidase-conjugated goat anti-mouse immunoglobulin G (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) for 60 min at 37°C; they were then visualized with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Effect of recombinant MRA on the course of infection in mice. Sf9 insect cells were seeded at a concentration of approximately 1×10^6 cells/ml in 80-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) and infected with recombinant baculovirus AcMRA or AcFB-D at a multiplicity of infection of 5 PFU per cell. Three days postinfection, the cells were harvested and washed three times with cold PBS. The pellets were resuspended with PBS and then frozen and thawed three times. The suspensions were sonicated for 30 s, and the protein concentration of the antigen was then adjusted to 10 mg/ml; the suspensions were then used for immunization of mice.

A total of 15 female BALB/c mice that were approximately 8 weeks old were divided into three groups. The first group was immunized intraperitoneally with 2 mg of the AcMRA-infected cell lysate premixed with complete Freund's adjuvant (Difco, Detroit, Mich.). Subsequently, on days 14 and 28, the same amounts of lysate with incomplete Freund's adjuvant (Difco) were injected via the same route into the mice. The second group was immunized with the control AcFB-D-infected cell lysate by using the method described above, and the third group did not receive any immunogens. These groups were designated the MRA, FB-D, and control groups, respectively. On day 20 after the final immunization, 5 μl of serum was collected from the tail veins of the immunized mice and used for determination of the antibody response. All of the mice were challenged intraperitoneally with 1×10^7 *B. microti*-infected erythrocytes, blood was collected daily from the tail veins, and the parasitemia was monitored with Giemsa-stained thin blood smears. The monitoring was done for a total of 20 days after the challenge infection. Differences in the percentage of parasitemia were statistically analyzed by using the independent Student *t* test and a *P* value of <0.05 as the value that represented a significant difference.

RESULTS

Characterization of the Maltese cross form and its MRA. During the developmental cycle of *B. microti*, three morphological forms were detected by light microscopy of the Giemsa-stained smears: the ring form (Fig. 1A, panel a), the pear-shaped form (panel b), and the Maltese cross form (panel c). In order to understand the role of the Maltese cross form in the growth cycle of *B. microti*, we determined the curve for its appearance by using the blood smears that were prepared periodically from the tail veins of *B. microti*-infected mice and compared it with the curve for development of parasitemia. As shown in Fig. 1B, the percentage of the Maltese cross form in the peripheral blood increased rapidly prior to the peak of parasitemia. At its maximum level, the Maltese cross form represented approximately 0.7% of the total merozoite forms.

Next, we carried out an IFAT to examine the localization of MRA in different forms of *B. microti* by using confocal laser scanning microscopy and MAb 2-1E. Positive staining by MAb 2-1E was observed in only about 1% of all forms of parasites, even at the peak of appearance of the Maltese cross form. As shown in Fig. 2, MAb 2-1E was found to be reactive mainly with the parasites undergoing transformation into the Maltese cross form (Fig. 2b and c) and subsequent division (Fig. 2d), as well as with extracellular merozoites (Fig. 2e). However, the MRA also appeared in a small percentage (about 1%) of all ring forms (Fig. 2a).

Cloning of the MRA gene. A *B. microti* cDNA expression library was screened with MAb 2-1E, and five cDNA clones were obtained. The nucleotide sequences of the cDNA inserts were determined and aligned by using the identical sequences. The results indicated that all of the cDNA clones had a common nucleotide sequence and could be considered members of one cDNA group whose members had the same origin. One cDNA clone, which contained the longest cDNA insert, was

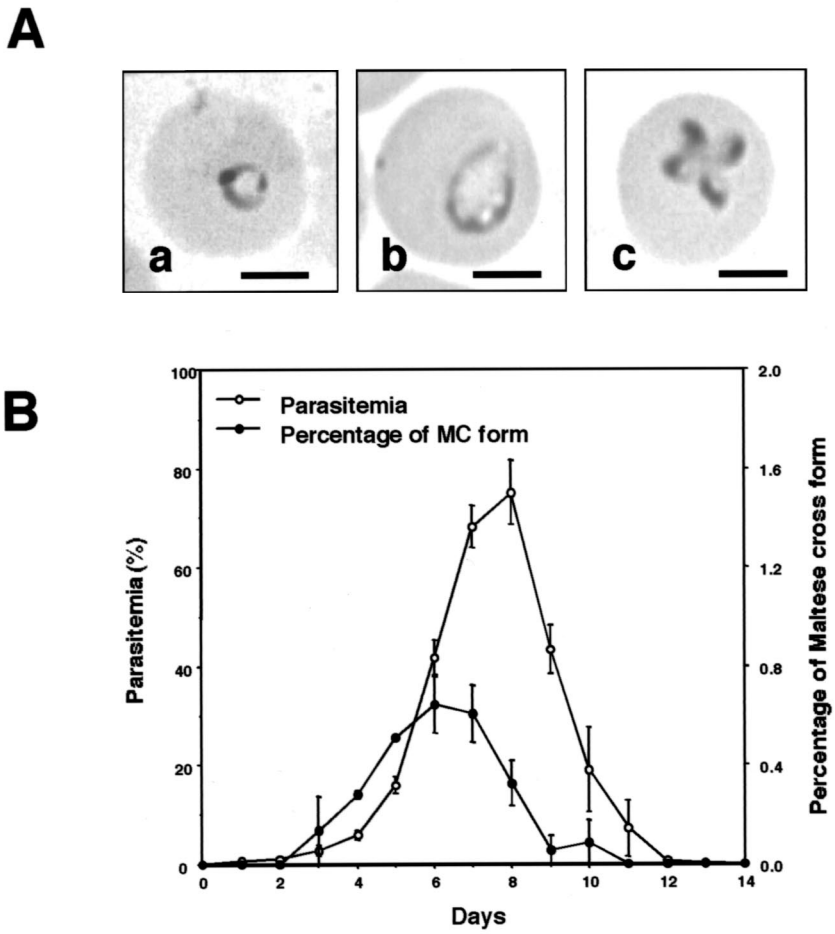

FIG. 1. (A) Micrographs of intraerythrocytic *Babesia* merozoites in Giemsa-stained blood smears. The ring (panel a), pear-shaped (panel b), and Maltese cross (panel c) forms were observed in the *B. microti*-infected murine erythrocytes. Bars = 2 μm. (B) Appearance curve for the *B. microti* Maltese cross (MC) form. Three female BALB/c mice that were approximately 8 weeks old were each infected intraperitoneally with 1×10^7 *B. microti*-infected erythrocytes. The parasitemia and the percentage of the Maltese cross form in infected erythrocytes were monitored daily by examining blood smears periodically prepared from blood from the tail veins. Each symbol indicates the mean, and the error bars indicate the standard errors.

chosen for subsequent analyses (GenBank accession number AB079857). The 5,382-bp insert of the cDNA clone did not have an available start codon at the 5′ terminus and had an incomplete open reading frame designated the MRA gene which encoded 1,739 amino acid residues with a predicted molecular mass of 192,788 kDa (Fig. 3A). Next, we searched for proteins with homology to the deduced amino acid sequence encoded by the MRA gene by using the National Center for Biotechnology Information database. The deduced sequence showed partial but significant identity (36% identity in a 135-amino-acid overlap) to the BMN 1-15 protein of *B. microti* (GenBank accession number AF206525) (Fig. 3B), which has been reported to be an immunodominant antigen that induces humoral immunity during *B. microti* infection in humans (17).

The cDNA sequence of the MRA gene does not contain any *Hin*dIII and *Pst*I cleavage sites, but it contains one *Xba*I and two *Eco*RI sites. In Southern blot analyses, the MRA gene probe recognized one band at approximately 17.5 kbp when the preparation was digested with *Hin*dIII and one band at approximately 15.2 kbp when the preparation was digested with *Pst*I (Fig. 4A, lanes 1 and 2). It also recognized two bands at approximately 3.0 and 3.9 kbp (lane 3) when the preparation was digested with the *Xba*I and three bands at approximately 0.8, 3.4, and 4.1 kbp (lane 4) when the preparation was digested with *Eco*RI. Additionally, to confirm that the MRA gene is really encoded on the genome of *B. microti* and is not of mouse origin, we carried out PCR analyses in which a pair of primers was designed to amplify a 3,404-bp DNA fragment from the MRA cDNA clone. As shown in Fig. 4B, approximately 3.4-kbp DNA fragments were detected with both the DNA extracted from the *B. microti*-infected blood (lane 2) and the MRA cDNA clone (lane 3). On the other hand, no band was detected when the DNA extracted from normal mouse blood was used (lane 1). These genomic analyses indicated that a single copy of the MRA gene is present in *B. microti.*

Expression of the MRA gene product and its role in protection against challenge infection in mice. In the next experiment, two kinds of recombinant baculoviruses were constructed for expression analysis. One of these baculoviruses,

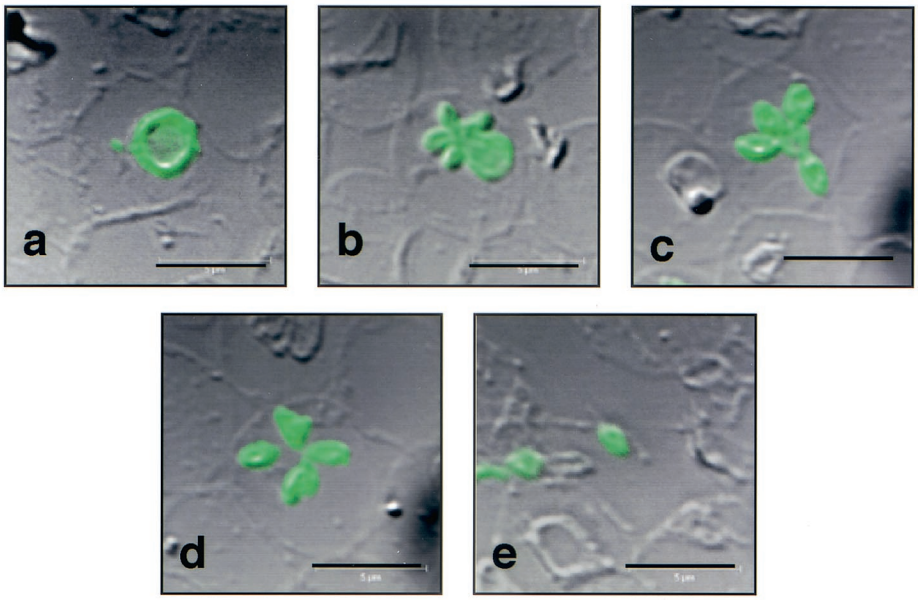

FIG. 2. IFAT performed by using confocal laser scanning microscopy. Methanol-fixed smears of the *B. microti*-infected erythrocytes were incubated with MAb 2-1E. The MAb-antigen reaction (green) was visualized with the FITC-conjugated secondary antibody. Bars = 5 μm.

AcMRA, was a recombinant virus carrying the MRA gene downstream of the viral polyhedrin promoter, while the other, AcFB-D, was a control virus without any inserted genes. In an IFAT with the immune serum collected from a *B. microti*-infected mouse, certain fluorescence was observed in the AcMRA-infected insect cells (Fig. 5A, panel a) but not in the AcFB-D-infected cells (panel b), indicating that the MRA gene product is immunodominant against *B. microti* infection. Additionally, in the lysate of AcMRA-infected insect cells, four polypeptides with molecular masses of approximately 54, 56, 92, and 178 kDa were recognized with MAb 2-1E in a Western blot analysis (Fig. 5B, lane 2). In contrast, no signal was detected in lysates of mock- and AcFB-D-infected cells (lanes 1 and 3). In the lysate of purified *B. microti* merozoites, only 89 kDa of the MRA was clearly detected (lane 4).

In order to examine the protective effect of the MRA gene product, mice were immunized with recombinant MRA and challenged with *B. microti*. All mouse sera from the MRA-immunized group specifically recognized the Maltese cross form of *B. microti* in blood smears (Fig. 6a), while sera from the FB-D group did not show any reaction (Fig. 6b). After the challenge infection, all mice in the control group showed typical development of high parasitemia, in which the parasitemia increased rapidly and reached the maximum value (approximately 44%) on day 8 (Fig. 7). Additionally, the parasites were detectable until the 19th day after challenge. On the other hand, development of parasitemia was significantly prevented in the MRA group, in which the maximum level of parasitemia was approximately 2% on day 6 and the parasites completely disappeared after the 9th day. In the FB-D group, development of parasitemia was also inhibited, but the maximum parasitemia was approximately 15% on day 7. The parasites were detectable until the 19th day, as in the control group. Consequently, the MRA group showed significantly lower parasitemia ($P < 0.05$) from day 6 to day 11 after infection than the control and FB-D groups. This challenge experiment was repeated, and the significant protective effect of the recombinant MRA was confirmed.

DISCUSSION

The Maltese cross form is known to be characteristic of *B. microti*, but its role in the development or reproduction of merozoites has not been understood. The present study showed that the percentage of the Maltese cross form increased rapidly in the peripheral blood of infected mice prior to the peak of parasitemia, suggesting that the Maltese cross form is involved in the rapid development of parasitemia. As determined by confocal laser scanning microscopy, the reactive forms were remarkably rare and were observed in only 1% of all forms of the parasite. MRA recognized mainly the Maltese cross form and the subsequently dividing merozoites. In transforming forms and their budding small progeny, the MRA was always located in the cytoplasm. In the ring form, MAb 2-1E was found in some of the structures (1%). The results of the IFAT and the correlation between the appearance of the Maltese cross form and rapid development of parasitemia suggest that a small population of the ring form transforms into the Maltese cross form and produces four progeny, thus contributing to the rapid development of parasitemia. However, it is necessary to identify by immunoelectron microscopy where the MRA is synthesized, translocated, and then accumulated in order to understand the precise role of MRA in the development of parasitemia.

A single copy of the isolated MRA gene was present in the *B. microti* genome. The deduced amino acid sequence of MRA showed partial similarity to the sequence of BMN 1-15 of *B. microti.* Recombinant BMN 1-15 was recently introduced as a target antigen for serological diagnosis of *B. microti* infection (17). Although MRA and BMN 1-15 are not identical, the

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NSAPGAFQEIVYLQIIGPLLFAKYNYNDIFSETEDTKNVKVAETKESSYLTHPLTSLLLSGYSAAYPLKFVESLITEIKHHSSNPPKQDYTVPFSALRSF  100
DELNLDEATVESLVSPLEEIKTKLVDTSNKPLTTIPEANVNSIFTSIRKFLQVSETRTYELLSLILAPANIFDDGNKYFNILPKYLTDVSVDLKSTTIPE  200
QVLFKDGIYKLKDQFKLSHKMAPTFGSVPLQIPINSAAVQINVKELDDNTKMAAIILDDLSKLTLEKVILSETPTVVGLTHMPPQNPLALLASIFVNVNI  300
TADGIIEGYVDLDLNSRTGNFFCALELTDLTNSIKYAMHDTEFINLPKLNVLALKFGNQLVEVKGTNKVFATFDENLDSVEIITFHEDGTKTSKFFSKEF  400
LGSMIPFTPYVPNYIEYACNGSMPCSPWITNHIHNKRLINFEVPIGILFEDKYPWIFLNKTKLHKDVAYSFYGNEHCVPNTTGLLLEGINDDRTKKVHEL  500
YKNEPSTTGSESADSKSASAGNATGPTTQTSTDDGRSGGAQSNVNIADKLQVKLKENDHPFPTPERNIPNCRIESIHFYIDNVVRHNAAFGEITIGSLTL  600
PAFSVGFIHSIFLEKVIKDHRNFISVGIITNYGPSGDYELLKYMQLEYGKKDFVLVSGPEVTADYIRSGLTKHKISFKQNEPTGLTGFETNYKESVHSNG  700
FLRPTNKMVTLFYTNDESEVKVSKLYSVEDGKHGVTYSIYGAFPVANASPNAGDISSIASVLNAMNYINRLLTTTYSALNALAPSNGTADSGSQVPDIRI  800
KNYLSKLSPDVVKPALSTLLKLLTGIMLKDAASTTSAVHTLSRFNTFIAFLNDKNNADYMKSAVIDDNVRAKFVEITNELIVGLEFVLDNIFKHHHMQTN  900
DEKVKTTLSSLMLTFRYINHIRHLINDVDLKVNPRMMMSNQTSIVANTNATGQNGHSTDGSQGNNLRATPQSSSGSEKAVEQTKIVTPSEPTPAANTQSIK 1000
ATTELGLVGHITKPSETSALTNFIDALCVELQAHIPQERAKPTANAADVTTGTTGTTGTTGKSEDTAEPKSDVTNQVTKFTTIASFDEKFKKLALTEDAS 1100
TAKCRKLVIDALNELKTTSTTTNGGDTNNSVKVENLLNIVLKLNNRIESWSSMLINHSFPKLCDSFVKVPTFSLPYFTTREVDITNMLSHISVKYYDGKF 1200
TIDPSEKDSRHTLAPNVTGHDLSLLHQLPISMITVTSPQQQKLTSCVDHGSDFTISSAAGKTTYTNKSKFLLHFKSSVETLKNFERAKFALADMIDTDPK 1300
SEILKIYESTLNDMKTTAGLLEGELKTMSITVIPFTDFITNAPVVNASEGKTTSINDIKTGFNYLARAITWYISSLLPTKFDQTNPESSSPPIEYKINLL 1400
SLNTAKSLLILEDLISSNNFIQITPMLEKTSEKTITKPVMDTNDGNIDKSKWTLMKLLNSGYLVDANMLCKKLINIFNASTKYTNAVKDKEIKDVDFDTT 1500
GMSDESRQLVVVLLTKIIRDLEIGHMITNKCKIRYNNIISRISNILLQEVIDIINRAYCTDIEKANHKPNIFNYPAFLEPIDIDILSPTVPEHVRFDNGV 1600
FKLTGSRKIELKLRPKVGSKYLEVSPHVAIIQVSVVDGIINVYGDDYHKITVQQFDMNGNIIRGGAFAVAHPFAQLAQAVALSVDDKTQIQNLQTSSDDD 1700
LFKKNISTYYENQIKVNQTTIKAQLKQTTPNDSTIILYP 1739
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B

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                                                    10        20        30
MRA                                              NSAPGAFQ-EIVYLQIIGPLLFAKYNYNDIF
                                                  : :. .  ...::: :  ... . .:::::
BMN 1-15  ISFDMLGTQKNKSSPLGKIGTSMDDIIAMFSNPNMYLVKVAYLQAIEHIFLISTKYNDIF
         300       310       320       330       340       350

                  40        50        60        70        80        90
MRA       SETEDTKNVKVAETKESSYLTHPLTSLLLSGYSAAYPLKFVESLITEIKHHSSNPPKQDY
           . : : .  :   :  .:.     :..:: : ..   :.:.:.::..:: :: .
BMN 1-15  DYTIDFS--KREATDSGSF-----TDILL-GNKVKESLSFIEGLISDIKSHSLKAGVTG-
         360       370             380       390       400

                 100       110       120       130       140
MRA       TVPFSALRS--FDELNLDEATVESLVSPLEE--IKTKLVDTSNKPLTTIPEANVNSIFTS
           .  :.: .  :::::::.::...::.::.   :. : .  : : ....:
BMN 1-15  GISSSSLFDEIFDELNLDQATIRTLVAPLDWPLISDKSLHPSLKMVVVLPGFFIVP
         410       420       430       440       450       460
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FIG. 3. (A) Deduced amino acid sequence encoded by the MRA gene (GenBank accession number AB079857). The sequence region showing significant identity to the sequence encoded by the BMN 1-15 gene is indicated by underlining. (B) Alignment of the deduced amino acid sequence encoded by the MRA gene with the amino acid sequence of BMN 1-15 of *B. microti* (GenBank accession number AF206525). Amino acid gaps are indicated by dashes, amino acid identities are indicated by colons, and conservative changes are indicated by dots.

MRA gene also produces an immunodominant antigen that induces humoral immunity against *B. microti* infection (Fig. 5). In a baculovirus expression analysis, four kinds of polypeptides that reacted with MAb 2-1E were synthesized. Although the largest polypeptide (178 kDa) was considered to be the original product, the MRA gene product seems to easily undergo a diverse range of processing after transcription in infected insect cells. In a Western blot analysis performed with purified *B. microti* merozoites, an approximately 89-kDa protein was recognized with MAb 2-1E and was thought to correspond to the 94-kDa protein described previously (20). In a previous study, MAb 2-1E recognized two proteins with molecular masses of approximately 94 and 180 kDa in the merozoite extract (20), while the larger 180-kDa protein could not be detected well in this experiment. Detection of *B. microti* MRA might be influenced by the blood stage collected, and native MRA also seems to undergo proteolytic processing, like the recombinant MRA described above. The immune sera raised with the recombinant MRA specifically recognized the MRA in the *B. microti*-infected blood smears in IFAT, assuming that the MRA unfailingly appears at the Maltese cross form stage and

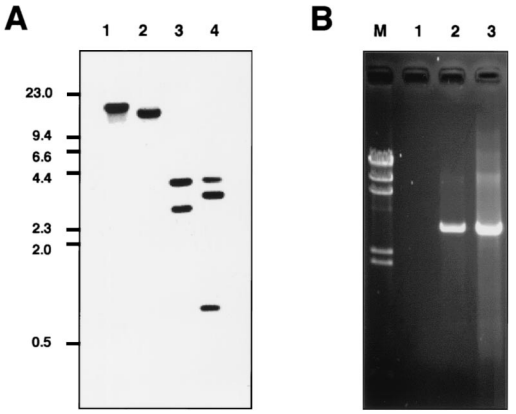

FIG. 4. (A) Southern blot analyses. The *B. microti* genomic DNA was digested with *Hin*dIII (lane 1), *Pst*I (lane 2), *Xba*I (lane 3), or *Eco*RI (lane 4), and the separated DNA fragments were hybridized with the MRA gene probe. The positions of λ *Hin*dIII DNA size markers (in kilobase pairs) are indicated on the left. (B) PCR amplification of the MRA gene in the DNA extracted from normal blood (lane 1) or *B. microti*-infected blood (lane 2) and cDNA clone of the MRA gene (lane 3). Lane M contained λ *Hin*dIII DNA size markers.

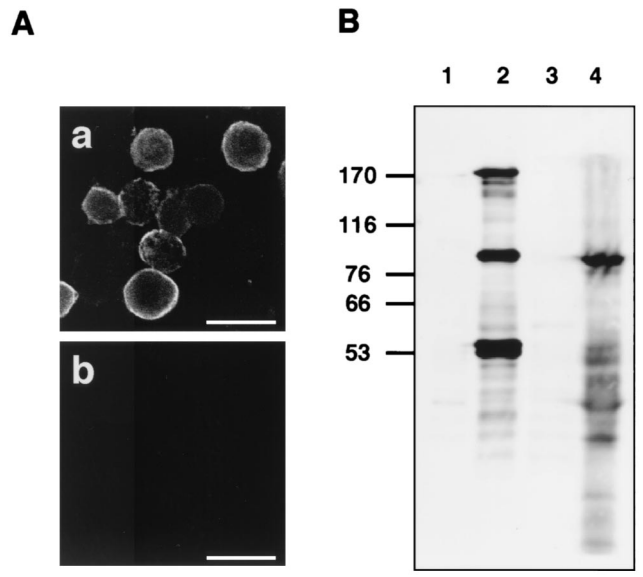

FIG. 5. (A) IFAT. Methanol-fixed smears of AcMRA-infected (panel a) or AcFB-D-infected (panel b) Sf9 cells were incubated with immune serum collected from a *B. microti*-infected mouse. The MAb-antigen reaction (white) was visualized with the FITC-conjugated secondary antibody. Bars = 20 μm. (B) Western blot analyses. The antigens prepared from noninfected (lane 1), AcMRA-infected (lane 2), or AcFB-D-infected (lane 3) Sf9 cell lysate or purified *B. microti* lysate (lane 4) were reacted with MAb 2-1E and visualized with an ECL kit. The positions of the molecular mass standards (in kilodaltons) are indicated on the left.

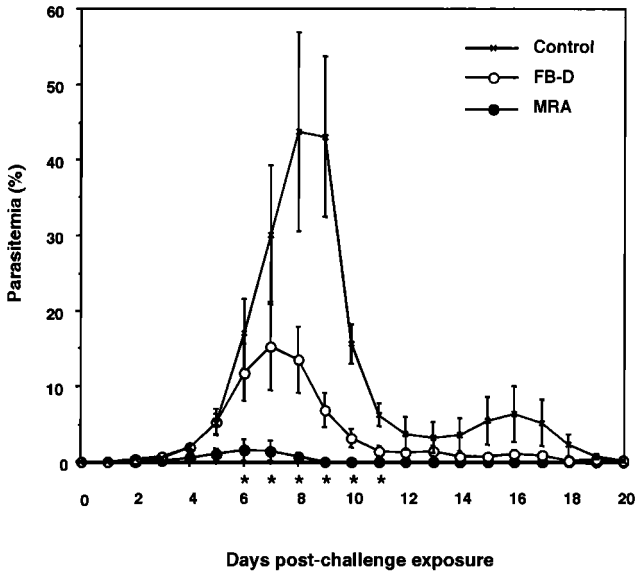

FIG. 7. Vaccine efficacy of the recombinant MRA against *B. microti* challenge. The five mice in the MRA group and the five mice in the FB-D group were immunized three times with AcMRA-infected cell lysate and AcFB-D-infected cell lysate, respectively, while the control group did not receive any immunogen. After challenge with 1×10^7 *B. microti*-infected erythrocytes, the developmental parasitemia in each group was monitored daily by examining blood smears periodically prepared from blood from the tail veins. Each symbol indicates the mean, and the error bars indicate the standard errors. The asterisks indicate the days on which there was a significant difference ($P < 0.05$) between the MRA and FB-D groups. The data are representative of two separate experiments.

participates in the transformation and division of merozoites. To understand the molecular and biochemical characteristics of MRA, especially how 94 kDa of native MRA is involved in the transformation into the Maltese cross form and the subsequent division, a study of the complete sequence of MRA cDNA is now in progress.

Immunization of mice with the recombinant MRA together with Freund's adjuvants induced significant protection against challenge infection with *B. microti*. Freund's adjuvants are known to induce strong cell-mediated immunity, as well as an antibody response in rodent malaria infection (3, 6). In *B. microti* infection, cell-mediated immunity, especially by $CD4^+$ T cells and gamma interferon, is known to play important roles in protection (11, 12). Passive immunizations with MAb 2-1E did not influence the development of parasitemia in the *B. microti*-infected mice (20), and no reaction was detected in intraerythrocytic and extracellular merozoites or around the infected erythrocytes in IFAT when the infectious blood was preincubated with MAb 2-1E before fixation with methanol (data not shown) (28). Therefore, the cell-mediated immunity induced by a combination of recombinant MRA and adjuvants might inhibit reproductive expansion of *B. microti* merozoites in mice. In contrast, merozoite development in the FB-D group was also inhibited, but only partially. Immunization with only Freund's adjuvants was reported to induce a partially protective effect for *B. rodhaini* infection (10). This effect has been considered to be due to nonspecific activation of macrophages (10). It would be interesting to determine why such a rarely detected antigen can induce protective immunity, and further studies of this phenomenon should shed light on new strategies for preventing protozoan infections.

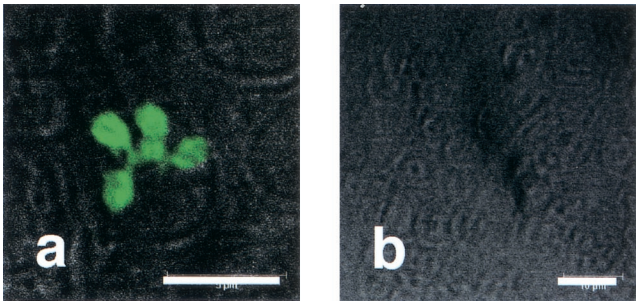

FIG. 6. IFAT performed by using confocal laser scanning microscopy. Methanol-fixed smears of *B. microti*-infected erythrocytes were incubated with immune sera of the MRA (a) or FB-D (b) group. The MAb-antigen reaction (green) was visualized with the FITC-conjugated secondary antibody. Bar = 5 μm (a) or 10 μm (b).

B. microti not only has zoonotic importance but also has been studied as a model for many other babesioses. Besides *B. microti*, *B. equi* (1, 24) and *T. parva* (5) are also known to have Maltese cross forms, which are tetrads, during the reproductive cycle in the erythrocytic stage. By using the nucleotide sequence or the antigenicity of *B. microti* MRA, it might be possible to isolate the homologous MRA genes from the cDNA libraries of these parasites (8). In particular, because the severity of *B. equi* infection is also associated with the

erythrocytic stage, as is infection with *B. microti*, and causes enormous losses in the horse industry (22), our report should be valuable for the development of protective measures against equine babesiosis in the future. In conclusion, the present findings indicate the key role of the Maltese cross form in the reproduction of *B. microti* and its potential application in prophylaxis against human and equine babesioses.

ACKNOWLEDGMENT

This work was supported by grants-in-aid for scientific research from the Japan Society for the Promotion of Science.

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Editor: W. A. Petri, Jr.