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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

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Abstract

During an asexual growth cycle of *Babesia* parasites in a natural host, the extracellular merozoites invade (i.e., attach to, penetrate, and internalize) the host erythrocytes (RBC) via multiple adhesive interactions of several protozoan ligands with the target receptors on the host cell surface. After internalizing the host RBC, they asexually multiply, egress from the RBC by rupturing the host cells, and then invade the new RBC again. In the invasion stage, several surface-coating molecules of merozoites might be involved in the initial attachment to the RBC, while proteins secreted from apical organelles (rhoptry, microneme, and spherical body) are proposed to play roles mainly in erythrocyte penetration or internalization. On the other hand, several components located on the surface of the RBC, such as sialic acid residues, protease-sensitive proteins, or sulphated glycosaminoglycans, are identified or suspected as the host receptors of erythrocyte invasion by *Babesia* parasites. The detailed molecular interactions between *Babesia* merozoites and the host RBC are incompletely understood. In this review, these identified or suspected molecules (protozoan ligands/erythrocyte receptors) are described by especially focusing on *Babesia bovis*.
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1. Introduction

Babesia parasites are tick-transmitted intraerythrocytic protozoa in the phylum Apicomplexa. They infect a wide variety of wild and domestic animals, and the majority of them are responsible for enormous economic losses to the livestock industry worldwide (Dewaal, 2000; Kuttler, 1988; Brown and Palmer, 1999). Moreover, some are important major etiologic agents of human babesiosis (Homer et al., 2000). Unlike *Plasmodium* parasites, which require hepatocyte invasion and multiplication prior to the erythrocyte stage, sporozoites of some *Babesia* parasites, such as *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*, *Babesia canis*, *Babesia caballi*, and *Babesia ovis*, can directly invade the host erythrocytes

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(RBC) and undergo an asexual growth cycle in the erythrocyte stage (Mosqueda et al., 2002a,b; Hoyte, 1965, 1971).

The process of erythrocyte invasion by *Babesia* parasites is considered to be similar to that by *Plasmodium* species, which has been well studied (Igarashi et al., 1988; Bannister and Mitchell, 2003; Preiser et al., 2000). During an asexual growth cycle in a mammalian host, extracellular merozoites invade (i.e., attach to, penetrate, and internalize) the host RBC and asexually multiply in the invaded RBC. After the divided trophozoites egress from the RBC by rupturing the host cells, the progeny merozoites invade new RBC again (Yokoyama et al., 2002, 2003; Kumar et al., 2004). The erythrocyte stage of *Babesia* parasites results in severe clinical symptoms, such as: high fever, anemia, hematuria, and hemoglobinuria, in the infected hosts (Homer et al., 2000). A considerable effort has so far been directed to block the asexual growth cycle with the intent of developing control strategies, including effective vaccination and chemotherapy against babesiosis (Wright et al., 1992; Dalrymple, 1993; Palmer and McElwain, 1995; Brown and Palmer, 1999; Jenkins, 2001; Yokoyama et al., 2003; Nagai et al., 2003; Combrink et al., 2002; Bork et al., 2003a,b,c, 2004). However, the molecular interactions between *Babesia* merozoites and host RBC and their biological roles are not thoroughly understood. Therefore, a full understanding of the basic molecular mechanisms of the asexual growth cycle, particularly the process of erythrocyte invasion by merozoites, is required for the development of preventive measure against babesiosis.

Apicomplexans utilize several protozoan molecules (ligands) in their invasion process (Preiser et al., 2000; Sam-Yellowe, 1996). Extracellular merozoites reversibly attach to the host RBC, re-orient to bring the apical organelles close to the attachment interface, and then penetrate the RBC membrane through various molecular interactions of the protozoan ligands with the target receptors of host cell surface (Dubremetz et al., 1998; Soldati et al., 2001). During the process, some of the surface molecules coating the merozoites may function as host-recognizing factors, while rhoptry and microneme products may be secreted from the organelles of merozoites to the point of membrane invagination in order to make tight junctions to the host RBC and then accomplish their penetration and subsequent internalization within the RBC (Preiser et al., 2000; Sam-Yellowe, 1996). Therefore, there must be corresponding host receptors that are probably located on the surface of the host RBC. Although the morphological observations during host cell invasion appear to be similar among apicomplexans, several molecular events vary, and some are unique, depending on the genus they belong to (Preiser et al., 2000; Sam-Yellowe, 1996; Igarashi et al., 1988).

In this review, we introduce several identified or suspected molecules (protozoan ligands/erythrocyte receptors) that are involved in the erythrocyte invasion by *Babesia* parasites. Additionally, this review focuses on *B. bovis*, which is highly pathogenic in bovines (Dewaal, 2000; Kuttler, 1988), because the parasite has been studied in the most detail among the family of *Babesia* parasites.

2. Protozoan ligands for the RBC

2.1. Surface-coating molecules of extracellular merozoites

At an initial step of erythrocyte invasion, *Babesia* parasites are considered to utilize their surface molecules coating the extracellular merozoites in order to effectively attach to the target RBC as demonstrated for malaria parasites (Preiser et al., 2000; Holder et al., 1999). In *B. bovis*, merozoites are known to possess at least five (glyco-) proteins on their surfaces, and these surface molecules belong to a family of variable merozoite surface antigens (VMSA) (Florin-Christensen et al., 2002; Hines et al., 1989; Cowman et al., 1984; Jasmer et al., 1992a). The members of the VMSA family consist of merozoite surface antigen 1 (MSA-1: 42 kDa), MSA-$2a_1$, MSA-$2a_2$, MSA-2b, and MSA-2c, which are defined by an amino-terminal hydrophobic signal sequence, a hydrophilic central region, and a conserved carboxy-terminal region containing a glycosyl-phosphatidylinositol (GPI) anchor signal sequence (Jasmer et al., 1992a; Hines et al., 1992; Florin-Christensen et al., 2002). Interestingly, they are expressed on the surfaces of not only merozoites but also tick-derived sporozoites (Goff et al., 1988; Mosqueda et al., 2002a,b; Palmer et al., 1991;

Reduker et al., 1989; Wilkowsky et al., 2003; Hines et al., 1989; Jasmer et al., 1992a). Essentially, all of the specific antibodies against the members of the VMSA family inhibit the merozoite invasion of bovine RBC, as well as the erythrocyte attachment by sporozoites (Hines et al., 1992, 1995b; Mosqueda et al., 2002a,b; Wilkowsky et al., 2003; Suarez et al., 2000). Based on their surface localization and the neutralizing activity of each antibody, the members of VMSA are proposed to play key roles in the initial attachment of merozoites and sporozoites during their invasion stage.

Allelic polymorphism, in which the surface-exposed epitopes of these VMSA proteins are not fully conserved among the geographic strains, is a feature of the *B. bovis* VMSA family (Suarez et al., 2000; Palmer et al., 1991; Florin-Christensen et al., 2002). Unlike MSA-1, which is encoded as a single copy gene in the genome of *B. bovis* without other VMSA genes in the flanking regions (Suarez et al., 2000), MSA-2 proteins (MSA-$2a_1$, MSA-$2a_2$, MSA-2b, and MSA-2c) are encoded by tandemly arranged genes within an 8.3-kb genomic locus of *B. bovis* (Florin-Christensen et al., 2002). It is considered that extensive rearrangement has resulted in the occurrence of these multiple genes that have 5′ or 3′ regions of sequence identity or close similarity (Cowman et al., 1984). Phylogenetic analysis of the VMSA genes suggested that the MSA-1 gene has an ancestral relationship to other MSA-2 genes (Florin-Christensen et al., 2002). MSA-$2a_1$ and MSA-$2a_2$ are the most closely related among the four MSA proteins, with 90% identity in the amino acid sequences, and have, respectively, 54 and 64% identities with MSA-2b and 25 and 26% identities with MSA-2c. MSA-2b and MSA-2c have 25% identity (Florin-Christensen et al., 2002). The structural differences among MSA-$2a_1$, MSA-$2a_2$, MSA-2b, and MSA-2c can be reflected by the presence of unique B-cell epitopes on each protein and the absence of a cross-reactive antibody (Florin-Christensen et al., 2002). However, the biological roles of these surface-coating antigens in erythrocyte invasion remain hypothetical, and the corresponding receptors of the RBC are still unknown.

Recently, the inhibitory effect of heparin was demonstrated on the growth of *Babesia* parasites (Bork et al., 2004). The multiplication of *B. bovis*, *B. bigemina*, *B. equi*, and *B. caballi* in in vitro cultures and of *B. microti* in vivo (mice) was significantly inhibited in the presence of or treatment with heparin. The treatment with heparin restricted the erythrocyte invasion by babesial merozoites. Furthermore, fluorescein isothiocyanate (FITC)-labelled heparin was preferentially found on the surface of extracellular merozoites. These findings indicate the presence of heparin-binding molecule(s) on the surface of extracellular merozoites and suggest a crucial role of these molecules in the erythrocyte invasion by merozoites (Bork et al., 2004). Due to the fact that the heparin-binding molecule and the MSA proteins have a similar localization, heparin might directly recognize the MSA(s) and competitively inhibit the essential molecular interaction of the MSA(s) with the host receptor(s), resulting in the blocking of erythrocyte attachment by the merozoites. Heparin binding might become a key to a better understanding of the function of MSA proteins.

2.2. *Rhoptry and microneme proteins of extracellular merozoites*

From their first attachment to their complete internalization during the invasion process, apicomplexan parasites secrete proteins from their apical organelles onto the merozoite membrane or into the environment outside the parasites (Preiser et al., 2000; Sam-Yellowe, 1996). These organelles are located at the anterior end of the parasites, where this unique complex of organelles is named an apical complex and comprises rhoptries, micronemes, and dense granules (spherical body in *Babesia* parasites) (Blackman and Bannister, 2001; Preiser et al., 2000; Dubremetz et al., 1998; Sam-Yellowe, 1996). Especially, secreted proteins from the rhoptries and micronemes are thought to play a central role in the sequence of events of cell invasion by apicomplexan parasites (Carruthers et al., 1999; Soldati et al., 2001; Menard, 2001; Tomley and Soldati, 2001).

In *B. bovis*, a 60-kDa rhoptry protein, designated as a rhoptry-associated protein-1 (RAP-1), had previously been identified (Suarez et al., 1991b; Dalrymple, 1993; Sam-Yellowe, 1996). The RAP-1 has an apical localization in the *B. bovis* merozoite (Dalrymple, 1993; Goff et al., 1988; Palmer et al., 1991; Reduker et al., 1989) and is clearly detectable as a rhoptry component by immuno-electron microscopy

(Suarez et al., 1993; Matsuo et al., in press). The RAP-1 of *B. bovis* is encoded by two identical and tandemly arranged RAP-1 genes (Suarez et al., 1998a,b) and has substantial sequence homology with the RAP-1 of other members in *Babesia* parasites (Dalrymple, 1993; Dalrymple et al., 1993; Suarez et al., 1991a). Additionally, the RAP-1 contains several immunogenic epitopes responsible for inducing host humoral immunity, and the structure is highly conserved among diverse isolates (Suarez et al., 1991a, 1993, 1994; Brown et al., 1996). Mosqueda et al. (2002a) reported that the *B. bovis* RAP-1 is also expressed in the sporozoites and that the specific antibodies can inhibit the sporozoite attachment to host RBC. Additionally, RAP-1 is detectable in all developmental stages of intracellular parasites (the ring and the subsequent pear-shaped forms) and extracellular merozoites during the asexual growth cycle of *B. bovis* (Yokoyama et al., 2002). An erythrocyte-binding assay with the lysate of merozoites proved the binding ability of the RAP-1 to bovine RBC, and the anti-RAP-1-specific antibodies prevented the interaction of the RAP-1 with bovine RBC. Furthermore, the antibodies significantly inhibited the proliferation of *B. bovis* in the in vitro culture (Yokoyama et al., 2002). These findings indicate that the RAP-1 has an imperative and functional obligation for the erythrocyte invasion by merozoites and sporozoites.

Recently, two invasion molecules, which are predicted as microneme products in the *B. bovis* merozoites, were newly identified by a research group at Utrecht University (Gaffar et al., 2004a,b). One is an apical membrane antigen 1 (AMA-1) homologue, and another is a thrombospondin-related anonymous protein (TRAP) homologue, respectively, designated as BbAMA-1 and BbTRAP in *B. bovis*. *Plasmodium* AMA-1 is known as a microneme protein that is secreted onto the surface of extracellular merozoites (Howell et al., 2003). The overall level of similarity of BbAMA-1 to *Plasmodium falciparum* AMA-1 is low, but the characteristic features of AMA-1, such as a transmembrane domain near the C terminus, a predicted short cytoplasmic C-terminal sequence, and an extracellular domain containing 14 cysteine residues, are typically conserved in the amino acid sequence of BbAMA-1 (Gaffar et al., 2004b). The BbAMA-1, with molecular weight of 82 kDa, was localized to the apical half of merozoites with the specific antibodies in the immunological analyses. Essentially, pre-incubation of extracellular merozoites with specific antibodies to BbAMA-1 reduced erythrocyte invasion by *B. bovis* (Gaffar et al., 2004a). On the other hand, the TRAPs of *Plasmodium* sporozoites are known to share several adhesive domains conserved in the microneme proteins of other apicomplexans and function as protozoan ligands for interacting with host cells (Sultan et al., 1997; Matuschewski et al., 2002). Like the *Plasmodium* TRAPs, the BbTRAP was also identified as a type 1 transmembrane protein containing von Willebrand A (vWFA) and thrombospondin type 1 (TSP-1) domains (Gaffar et al., 2004b). In the immunological analyses with the anti-BbTRAP-specific antibodies, the 75 kDa BbTRAP was located mainly at the apical side of merozoites. The specific antibodies were also found to inhibit erythrocyte invasion by *B. bovis* merozoite (Gaffar et al., 2004a). The TRAP of *P. falciparum* binds to sulphated glycosaminoglycans (GAGs) of human hepatocytes using the TSP-1 domain for their adhesion (Robson et al., 1995; Muller et al., 1993), while heparin has a binding affinity for the vWFA domain of TRAP (McCormick et al., 1999). Therefore, the BbTRAP might also play an important role as a protozoan ligand for GAG.

Since RAP-1, BbAMA-1, and BbTRAP are all detectable in the supernatant of a *B. bovis* culture, these proteins seem to function as secretory proteins during the erythrocyte invasion by merozoites (Johnson et al., 1997; Gaffar et al., 2004a,b). Identification of their target receptors is essential to understand the biological roles of these antigens at the stage of erythrocyte invasion.

2.3. Components of the spherical body

Intracellular protozoan parasites internalize the host cell environment by releasing parasite proteins into the cytoplasm of infected host cells during and after penetration in order to make the environment an optimal condition for the parasites. The secreted proteins involved in cell internalization play various roles, for instance, in parasite penetration, transportation, or association, or as an aid to parasite growth and viability (Ruef et al., 2000; Bannister and Mitchell, 2003). In the genus *Babesia*, instead of a dense granule, which is one of the major apical organelles in

apicomplexans, a unique organelle called a spherical body is present in the babesial apical complex (Potgieter and Els, 1977; Sam-Yellowe, 1996). In apicomplexan parasites, the dense granule proteins are considered to function in host cell internalization (Blackman and Bannister, 2001; Dubremetz et al., 1998). However, the internalization processes vary within the members of the phylum Apicomplexa. In particular, parasitophorous vacuoles (PV) of the *Babesia* species, as well as the *Theileria* species, rapidly disintegrate following the penetration step, unlike *Plasmodium* and *Toxoplasma* species, in which the PV formation must be kept during the internalization (Sam-Yellowe, 1996). Until now, three proteins, spherical body protein 1 (SBP-1: 77–80 kDa) (Hines et al., 1995a), SBP-2 (225 kDa) (Jasmer et al., 1992b; Dowling et al., 1996), and SBP-3 (135 kDa) (Ruef et al., 2000), have been identified in the spherical bodies of *B. bovis*. These proteins have putative signal peptides at the N-terminus and are released into the cytoplasmic face of the infected RBC membrane during the course of erythrocyte infection (Hines et al., 1995a; Dowling et al., 1996; Ruef et al., 2000). No consensus targeting sequences have been implicated for the dense granule proteins of other apicomplexans (Ruef et al., 2000). Although it has been proposed that the contents of the spherical bodies are involved in the dissolution of the PV membrane via the host cell association in *Babesia* parasites, further experiments are needed to identify their biological functions.

2.4. Vaccine development based on the invasion factors

Erythrocyte invasion is one of the most critical stages of the asexual growth cycle that is immunologically vulnerable. Extracellular merozoites are directly exposed to the host humoral immune components, while the antibody never reaches the parasites inside the parasitized RBC (Yokoyama et al., 2002). Therefore, the elucidation of the invasive process will lead to the successful identification of potential candidates applicable for the development of a babesial vaccine, like *Plasmodium* parasites, where an increased number of ligands involved in the invasive stage have been identified and some of them are being explored as candidate vaccine antigens (Moorthy et al., 2004; Mahanty et al., 2003). The main requirements for this type of vaccine are the inclusion of surface-exposed or functionally relevant antigens expressing B- and T-cell epitopes conserved among different strains. Additionally, the expression of MSA-1, MSA-$2a_1$, MSA-$2a_2$, MSA-2b, MSA-2c, and RAP-1 in both sporozoites and merozoites increased the likelihood of effective vaccination against a tick challenge using these antigens (Mosqueda et al., 2002a,b). Thus, the inhibition of the initial invasion of the RBC by sporozoites may be effective in the control of babesiosis. In addition, the MSA-2c and RAP-1 appear to be highly conserved among geographically distant *B. bovis* strains (Suarez et al., 1998a,b; Florin-Christensen et al., 2002; Wilkowsky et al., 2003). Therefore, these antigens have been targeted for the development of subunit vaccines against bovine babesiosis. However, while the vaccine utilities of recombinant proteins based on the invasion factors of *B. bovis* have so far been evaluated in vivo, complete protection has not yet been achieved. For instance, the *B. bovis* MSA-1 strongly induced the in vitro-neutralizing antibodies, as well as a $CD4^+$ T-lymphocyte response (Brown et al., 1993; Hines et al., 1995b; Suarez et al., 2000), but immunization with recombinant MSA-1 was not protective in cattle against subsequent challenge with virulent *B. bovis* (Hines et al., 1995b). Similarly, although the purified recombinant RAP-1 of *B. bovis*, as well as native *B. bigemina* RAP-1, were reported to be partially effective in inducing a protective immunity with regard to reduced parasitaemia in the vaccinated cattle (Wright et al., 1992; McElwain et al., 1991), a recent study by Norimine et al. (2003) found no evidence of protective immunity in spite of the immunization resulting in priming for strong cell-mediated responses (such as induction of interferon-γ and T-cell proliferation) and humoral responses (antigen-specific IgG secretion) against RAP-1 or the N-terminal half of RAP-1 upon challenge infection. Thus, development of effective vaccines based on the antigens purportedly involved in invasion processes of *Babesia* parasites has been difficult. Further identification and in vivo characterization of parasite molecules are needed to develop vaccine candidates that block invasion and induce of strong immunity, both of which can lead to protection. This is an active field of research for bovine babesiosis.

3. Erythrocyte receptors for *Babesia* parasites

3.1. Sialic acid residues

In previous studies (Kania et al., 1995; Zintl et al., 2002a; Gaffar et al., 2003), sialic acid residues of bovine RBC have been implied to function as a host receptor for bovine *Babesia* parasites (*B. bovis*, *B. bigemina*, and *B. divergens*) in the process of erythrocyte invasion. Pre-treatment of bovine RBC with neuraminidase (Nm) was shown to significantly decrease the susceptibility to erythrocyte invasion by the parasites. Additionally, *B. bovis* and *B. divergens* can invade not only bovine RBC but also human and other animal RBC in a sialic acid-dependent mechanism (Gaffar et al., 2003; Zintl et al., 2002a,b).

Sialic acids are terminal or subterminal non-reducing units attached by mainly α2–3 or α2–6 linkages to underlying galactose residues of many glycoconjugates and include the commonest structures, such as *N*-acetylneuraminic acid (Neu5Ac) and its 5-position-substituted variant from the acetyl to hydroxyl base, *N*-glycolylneuraminic acid (Neu5Gc); the latter is not easily detected in healthy humans but is often found in other mammals (Varki, 2001). The sialic acid residues on the host cells have been shown to play an important role in several aspects of microbial pathogenesis (Rogers et al., 1983; Higa et al., 1985; Hirmo et al., 1996). For instance, in *P. falciparum*, the erythrocyte-binding antigen-175 (EBA-175) has been demonstrated to bind to Neu5Acα2–3Gal-sequences of glycophorin A, which is present as an abundant sialoglycoprotein on the surface of human RBC (Orlandi et al., 1992). However, the importance of the type of sialic acid (Neu5Ac or Neu5Gc) or the linkage of sialic acid (α2–3 or α2–6 linkages) to its conjugate in the erythrocyte invasion by *B. bovis* remains unclear. Recently, we demonstrated that the pre-treatment of bovine RBC with Nm decreased the binding of α2–3-linked sialic acid-specific lectin, but there was no difference in the binding of α2–6-linked sialic acid-specific lectin (Okamura et al., submitted for publication). Additionally, the Nm treatment removed the α2–3-linked sialic acid residues from 97, 33, and 31 kDa of the surface proteins of bovine RBC. Furthermore, the addition of 3′-sialyllactose (Neu5Acα(2–3)Galβ(1–4)Glc) to the serum-free culture of *B. bovis* showed a more effective inhibition of the parasite growth than either Neu5Ac, Neu5Gc, or 6′-sialyllactose (Neu5Acα(2–6)Galβ(1–4)Glc). These results further confirmed the previous study by Gaffar et al. (2003), where the pre-incubation of merozoites with a mixture of 85% of the Neu5Acα(2–3)Galα(1–4)Glc isoform and 15% of the Neu5Acα(2–6)Galβ(1–4)Glc isoform decreased the erythrocyte invasion by *B. bovis*. Taken together, these findings suggest that the α2–3-linked sialic acid-containing membrane glycoprotein(s) plays a role as a host receptor in the erythrocyte invasion by *B. bovis* regardless of the type of sialic acid (Neu5Ac or Neu5Gc). Therefore, it is very interesting to identify the α2–3-linked sialic acid-containing membrane glycoprotein(s) and the protozoan ligand corresponding to the receptor.

3.2. Trypsin- or α-chymotrypsin-sensitive proteins

Kania et al. (1995) reported that pre-treatment of bovine RBC with trypsin significantly decreased erythrocyte invasion by *B. bigemina*, whereas α-chymotrypsin had little effect. On the other hand, erythrocyte invasion by *B. divergens* was not affected by the trypsin treatment, while pre-treatment with α-chymotrypsin led to a reduction in the parasite growth (Zintl et al., 2002a). Additionally, Gaffar et al. (2003) demonstrated the inhibitory effects of trypsin and α-chymotrypsin treatments in the erythrocyte invasion by *B. bovis*. These results indicate that there might be different host receptors sensitive to these proteolytic degradations or different pathways at the invasion step in the members of bovine *Babesia* species.

3.3. Sulphated glycosaminoglycan

As described above, heparin, a sulphated GAG, binds to the surface of babesial merozoites and inhibits the subsequent invasion of host RBC (Bork et al., 2004). Heparin is a highly sulphated form of heparan sulphate (HS) and is well known as an inhibitor of the blood coagulation system (Jaques, 1979). The heparin and HS are complex entities composed by anionic, linear mucopolysaccharides with alternating uronic acid and hexosamine residues, in which a limited set of monosaccharide units gives rise to a number of complex sequences by variable substitution with *O*-sulphate, *N*-sulphate,

and *N*-acetyl groups (Mulloy and Forster, 2000). Heparin is a biosynthetical component of mast cells and basophils, while HS is produced in most cell types (Hileman et al., 1998). Due to the sulphate and carboxylate residues, heparin is highly negatively charged and has a selectively high affinity for various molecules (Casu, 1985). The heparin has fully been used as a model GAG to study the HS interaction with its binding partners (Mulloy and Linhardt, 2001). A previous study with *Toxoplasma gondii* reported that FITC-labelled heparin was localized near the sub-apical region of extracellular tachyzoites (Botero-Kleiven et al., 2001). *T. gondii* surface antigen, SAG3, is known to show a heparin-binding property and mediate the attachment of tachyzoite to the cellular HS proteoglycan of host cells (Jacquet et al., 2001). In *P. falciparum*, heparin interacts with the circumsporozoite (CS) protein expressed on the surface of the sporozoite (Rathore and McCutchan, 2000). The binding of CS protein to cellular HS proteoglycans is required for sporozoite attachment to hepatocytes. It is noteworthy that the HS proteoglycans are utilized in the host recognition by some parasitic microbes (Rostand and Esko, 1997). Together with the fact that HS-like GAG is located on the surface of the RBC (Trybala et al., 1993; Xiao et al., 1996), babesial merozoites might recognize the HS-like GAG of the host RBC via the parasitic molecule(s) with an affinity for heparin.

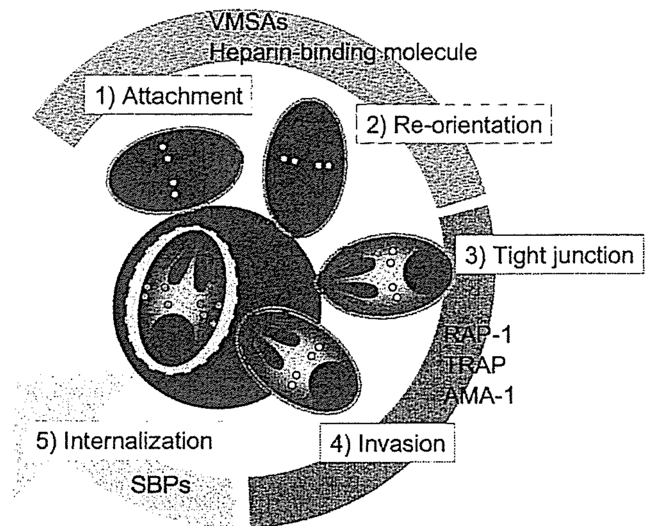

Fig. 1. Schematic representation of erythrocyte invasion process of *Babesia bovis*. First, the parasite attaches to the host RBC (1). The known or predicted protozoan ligands interacting with erythrocyte surface are VMSA family and heparin-binding molecule(s) that probably bind to HS-like GAG. After re-orientation (2), the parasite forms tight junction between erythrocyte surface and apical part (3) and starts invasion (4). In these steps, RAP-1 secreted from rhoptries and TRAP and AMA-1 from micronemes are probably involved in the interaction with erythrocyte surface. TRAP is considered to be a ligand for unknown GAGs on the RBC. Although corresponding receptors for RAP-1 and AMA-1 are not unclear, protein-based molecules, some of which must contain sialic acids, are also considered as erythrocyte surface receptors. Finally, the parasite is internalized within the infected RBC (5). SBPs are secreted from the spherical bodies to the erythrocyte cytoplasm, reportedly to lyse the PV.

4. Concluding remarks

The severity of babesiosis is associated with the asexual multiplication of causative parasites in the host RBC, leading to high parasitaemia followed by erythrolysis, anemia, and malaise in the infected animals. Therefore, it is highly desirable to understand the basic molecular mechanisms during the asexual growth cycle of *Babesia* parasites, particularly, the process of erythrocyte invasion by merozoites. Although, as described above, many studies have been carried out to identify the merozoite ligands/ erythrocyte receptors, the invasion mechanism of babesial merozoites is not fully understood (Fig. 1). Limited knowledge has hindered a successful development of an effective babesial vaccine or drug capable of restricting the growth cycle of parasites and completely eliminating the parasites from the host animal.

For understanding the invasion mechanism of merozoites, there still are many problems that should be resolved. First, we must set up a suitable method to precisely evaluate the invasion properties of babesial merozoites. Although an in vitro invasion assay, which uses free merozoites forcibly obtained by the application of high-voltage to the infected RBC (Franssen et al., 2003), is attractive, a further improved tool that can closely replicate the natural invasion of merozoites, for example, a combination of the synchronization of their growth cycle and purification of the functional extracellular merozoites, should also be constructed. Second, there is not much information about the protozoan ligands involved in the invasion of erythrocytes by babesial merozoites. Genome projects that are now in progress for some *Babesia* parasites

would help us with the effective identification of more protozoan ligands. Third, we must fully and precisely identify the receptors for *Babesia* parasites. For this purpose, more knowledge about the membrane components of animal RBC becomes essential. Fourth, we should demonstrate the interaction between the protozoan ligands and host receptors and the steps necessary for the links (e.g., merozoite attachment, re-orientation, penetration, or internalization) during the invasion stages. Finally, using the information on the morphological characteristics of invasive merozoites, we will be able to elucidate the sequence of events and biological roles of molecular interactions and identify the mechanism whereby *Babesia* parasites invade the host RBC.

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Calcium-ions are involved in erythrocyte invasion by equine *Babesia* parasites

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SUMMARY

Ethylene glycol *bis* (β-aminoethylether)-*N,N,N,N*-tetraacetic acid (EGTA) is a chelating agent capable of binding to positively-charged metal ions, including a calcium-ion (Ca^{2+}). Here, we demonstrated the inhibitory effect of the chemical on the *in vitro* asexual growth of the equine protozoan parasites, *Babesia caballi* and *Babesia equi*. The growth of both *B. caballi* and *B. equi* was significantly inhibited in the presence of EGTA (IC_{50} = 1·27 and 2·25 mM, respectively). Under microscopical observation, increased percentages of extracellular merozoites in the total parasites were detected in both of the cultures treated with high concentrations of EGTA. In contrast, further addition of Ca^{2+} to the EGTA-treated cultures prevented the parasites from clearing and the percentages of extracellular merozoites from increasing. As for *B. caballi*, an invasion test using high-voltage pulsing proved that EGTA has an inhibitory effect to their erythrocyte invasion. These results suggest that Ca^{2+} is involved in erythrocyte invasion by equine *Babesia* parasites.

Key words: *Babesia caballi*, *Babesia equi*, Ca^{2+}, EGTA, erythrocyte invasion.

INTRODUCTION

Babesia caballi and *Babesia equi*, in which *B. equi* is also described as *Theileria equi* (Mehlhorn and Schein, 1998), are the causative agents of equine babesiosis in tropical and subtropical regions, including Central and South America, Africa, Asia, and Southern Europe, leading to enormous losses in the horse industry (Schein, 1988). *Babesia* parasites invade, replicate in, and rupture erythrocytes (RBC) to repeat the asexual growth cycle, thereby causing severe clinical symptoms, such as haemolytic anaemia, icterus, fever, oedema, loss of body weight, and poor performance in the infected horses (Kuttler, 1988; Schein, 1988). Effective strategies for eradicating babesiosis should be performed on the basis of the biological mechanism of the asexual growth cycle of *Babesia* parasites.

Ethylene glycol *bis* (β-aminoethylether)-*N,N,N,N*-tetraacetic acid (EGTA) is a chelating agent capable of binding to positively-charged metal ions of various heavy elements, including Ca^{2+} and Zn^{2+} (Bers, 1982; Tatsumi and Fliss, 1994). In particular, the EGTA is known to chelate the Ca^{2+} more effectively than ethylenediaminetetraacetic acid (EDTA) (Schmid and Reilley, 1957). EGTA can not permeate the biological membrane of cells (Simons, 1991), so that it chelates only the extracellular ions. The Ca^{2+} functions as a major vital molecule in host cell infections by several protozoa. The attachment and invasion by *Plasmodium knowlesi* were greatly reduced or abolished by the presence of 2·5 mM EDTA or EGTA (Johnson *et al.* 1980). In *Toxoplasma gondii*, oscillations in the intracellular Ca^{2+} level of the parasite influence microneme secretion and prevent the parasite's gliding motility (Wetzel *et al.* 2004). In the bovine *Babesia* parasite, *Babesia bovis*, extracellular Ca^{2+} (1 mM) had a slight positive effect on their invasion (Franssen *et al.* 2003). However, no reports about *B. caballi* and *B. equi* in relation to Ca^{2+} chelation have been published. In this study, we investigated the inhibitory effect of EGTA on the *in vitro* cultures of *B. caballi* and *B. equi* and evaluated the invasion ability of *B. caballi* in the presence of EGTA by using high-voltage pulsing (Franssen *et al.* 2003). We discuss the vital role of extracellular Ca^{2+} in erythrocyte invasion by the parasites.

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MATERIALS AND METHODS

Parasites

U. S. Department of Agriculture (USDA) strains of *B. caballi* and *B. equi* were maintained in equine RBC in a microaerophilic stationary-phase culture system (Avarzed *et al.* 1997). The equine RBC were obtained from a healthy donor horse as previously described (Bork *et al.* 2003*a*). The culture media for *B. caballi* and *B. equi* were RPMI 1640 and

M199 (Sigma-Aldrich, Tokyo, Japan), respectively, both of which were supplemented with 40% normal equine serum, 60 units/ml penicillin G, 60 μg/ml streptomycin, and 0·15 μg/ml amphotericin B (Sigma, St Louis, MO, USA). For *B. equi* only, 13·6 μg/ml hypoxanthine (ICN Biomedical, Solon, OH, USA) was added to the medium (Nagai *et al.* 2003; Bork *et al.* 2003*a*; Ikadai *et al.* 2001). Infected RBC were mixed with freshly prepared RBC and then suspended in the culture medium at 10% packed cell volume in 24-well culture plates (Nunc A/S, Roskilde, Denmark). The *B. caballi* culture was incubated at 37 °C in 5% CO_2 in air, while the *B. equi* were cultured at 37 °C in 5% CO_2, 5% O_2, and 90% N_2. The overlaying growth medium was replaced daily with a fresh medium, and the infected RBC were subcultured every 3 days or when parasitaemia reached 5% (Bork *et al.* 2003*a*).

Chemicals

An EGTA stock solution of 1 M was prepared by dissolving 190·17 g of EGTA (WAKO Pure Chemical Industries, Osaka, Japan) with 500 ml of deionized distilled water (DDW) and subsequently adjusting the pH to 8·0 with 10 M NaOH. The stock solution was then sterilized by autoclaving and kept at room temperature until use. Working media containing the indicated concentrations of EGTA were prepared by diluting the stock solution with the culture medium and then adjusting the pH to 8·0 with 10 M NaOH again. A Ca^{2+} stock solution of 1 M was prepared by dissolving 73·5 g of $CaCl_2 \cdot 2H_2O$ (WAKO Pure Chemical Industries) with 500 ml of DDW. The solution was sterilized by filtrating with a 0·25 μm filter (Toyo Roshi, Tochigi, Japan). Working media containing the indicated concentrations of Ca^{2+} were prepared by diluting the stock solution with the culture medium.

In vitro *growth-inhibition test of EGTA*

The growth-inhibition test for EGTA followed the methods used for measuring drug activity as previously described (Bork *et al.* 2003*a*, *b*), with some modifications. Briefly, infected RBC were diluted with non-infected RBC to obtain 1% parasitaemia. The 50 μl of RBC mixture was subsequently suspended in 450 μl of the culture medium supplemented with EGTA, Ca^{2+}, or EGTA + Ca^{2+} at the indicated concentrations. The suspension was added into a 48-well culture plate (Nunc A/S) and then incubated in humidified multigas water-jacketed incubators at 37 °C for 3 days. In parallel, normal cultures, which were supplemented with the same volume of DDW instead of the chemical stock solution, were prepared as the control. All experiments were carried out in triplicate for each chemical. The culture medium was replaced daily with 450 μl of fresh medium containing the appropriate concentration of the chemical(s). The percentage of extracellular merozoites was calculated on day 1 of cultivation as the ratio of extracellular merozoites to the entire parasite population in approximately 1000 parasites.

In vitro *invasion test using high voltage pulsing*

The inhibitory effect of EGTA on erythrocyte invasion by *B. caballi* was evaluated by the invasion test using high voltage pulsing as described previously (Franssen *et al.* 2003) with some modifications. Briefly, *B. caballi*-infected RBC were suspended in an equal volume of *B. caballi* medium. The mixture of 400 μl was subjected to 5 intermittent (10 sec, 0 °C) high voltage pulses (1·5 kV, 400 O, 25 μF) in a Bio-Rad Gene Pulser II (Hercules, CA, USA) using a 0·2 cm pulser cuvette (Bio-Rad). The samples were then suspended with *B. caballi* medium or the media supplemented with EGTA at the indicated concentrations. After low centrifugation (700 *g*, 18 °C, 5 min), the pellet was resuspended with *B. caballi* medium or the indicated medium supplemented with EGTA, transferred to a 96-well plate (Nunc A/S) with non-infected RBC at 10% packed cell volume, and then incubated at 37 °C for 1 h. After this incubation, the numbers of infected RBC were counted in a total of 5000 RBC in Giemsa-stained smears, and the invasion efficiency was calculated as the percentage parasitaemia in the culture with EGTA compared to that in the normal medium control (100%). This experiment was performed in triplicate.

Statistical analyses

The values of a 50% inhibitory concentration (IC_{50}) of EGTA for both parasites were calculated on day 3 of cultivation after determination of curve fitting (Bork *et al.* 2003*a*, *b*). Differences in the percentage of parasitaemia and the percentage of extracellular merozoites were statistically analysed by use of an independent Student's *t*-test with $P < 0{\cdot}05$ representing a significant difference.

RESULTS

Inhibitory effect of EGTA on the growth of B. caballi

Inhibitory effects of EGTA were significant for the *in vitro* growth of *B. caballi* at concentrations of 1·5, 2 and 2·5 mM ($P < 0{\cdot}05$) as compared to the control growth, whereas concentrations of 0·5 and 1 mM did not show any effect (Fig. 1A). The IC_{50} value of EGTA was determined as 1·27 mM. Under microscopical observation, high percentages of extracellular merozoites were detected in the total

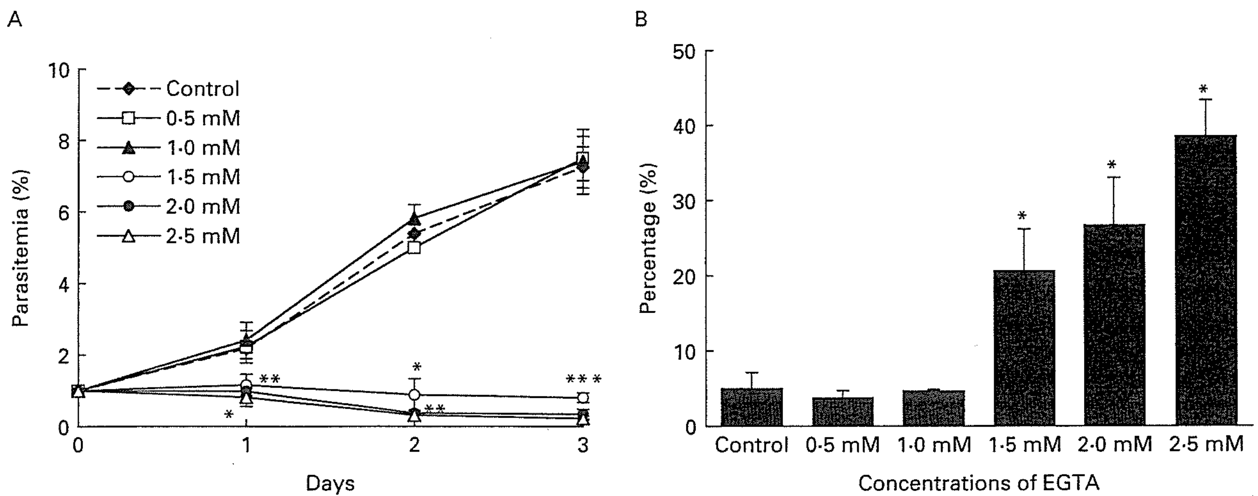

Fig. 1. Inhibitory effect of different concentrations of EGTA on asexual growth of *Babesia caballi* (A) and percentage of extracellular merozoites on day 1 of cultivation (B). Each value represents the mean ± standard deviation (S.D.) in 3 separate trials. The asterisks indicate significant differences ($P < 0·05$) between the chemical-treated and control groups.

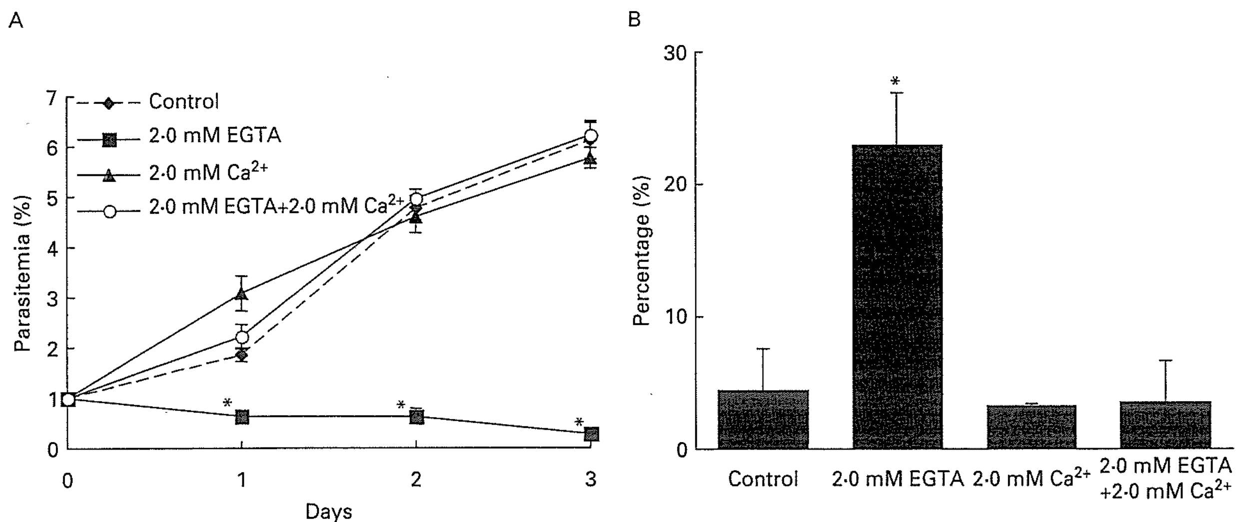

Fig. 2. Effect of addition of Ca^{2+} into the EGTA-treated *Babesia caballi* culture on the parasite growth (A) and percentage of extracellular merozoites on day 1 of cultivation (B). Each value represents the mean ± S.D. in 3 separate trials. The asterisks indicate significant differences ($P < 0·05$) between the chemical-treated and control groups.

parasites on day 1 of cultivation in the presence of 1·5, 2, and 2·5 mM EGTA (Fig. 1B). These percentages were significantly higher ($P < 0·05$) than that of the control. Any morphological changes were not found in the EGTA-treated parasites.

Although the growth of *B. caballi* was completely inhibited in the presence of 2 mM EGTA, addition of 2 mM Ca^{2+} recovered the normal growth pattern even in the presence of 2 mM EGTA (Fig. 2A). No difference in growth pattern was detected between the culture containing 2 mM Ca^{2+} without EGTA and the control culture. Although an abnormally high percentage of extracellular merozoites was produced in the culture containing 2 mM EGTA, the addition of 2 mM Ca^{2+} reduced their percentages to the same levels seen in the control culture (Fig. 2B). The percentage of extracellular merozoites in the culture containing 2 mM Ca^{2+} without EGTA showed almost the same value as that of the control culture (Fig. 2B).

Inhibitory effect of EGTA on the growth of B. equi

The *in vitro* growth of *B. equi* was significantly inhibited in the presence of EGTA at concentrations of 2·5 and 3 mM ($P < 0·05$) as compared to the control growth (Fig. 3A). The IC_{50} value was determined as 2·25 mM. The percentages of extracellular merozoites were also increased in the cultures of *B. equi* which contained 2·5 and 3 mM EGTA (Fig. 3B).

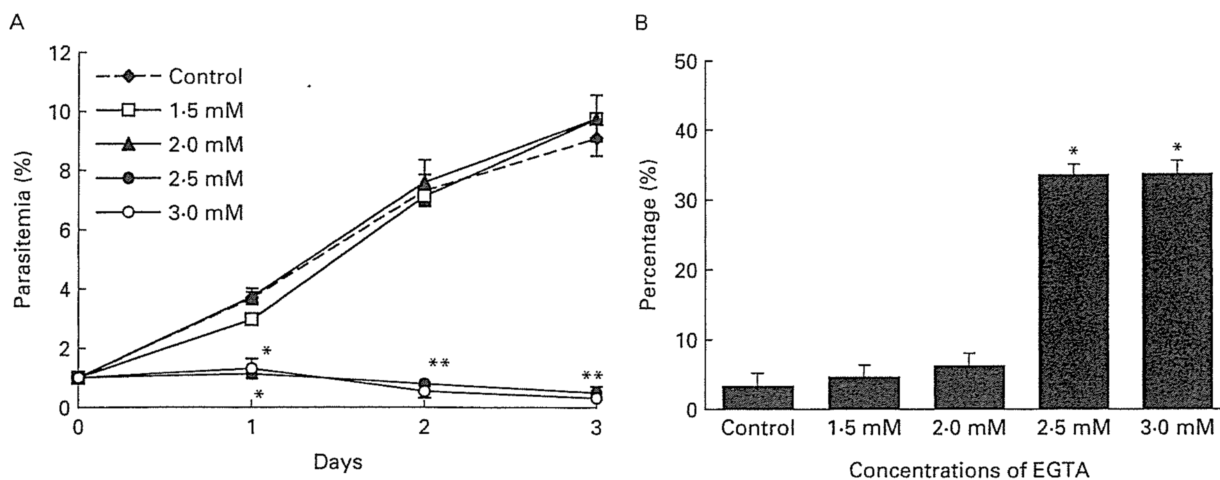

Fig. 3. Inhibitory effect of different concentrations of EGTA on asexual growth of *Babesia equi* (A) and percentage extracellular merozoites on day 1 of cultivation (B). Each value represents the mean ± standard deviation (S.D.) in 3 separate trials. The asterisk indicates significant differences ($P<0{\cdot}05$) between the chemical-treated and control groups.

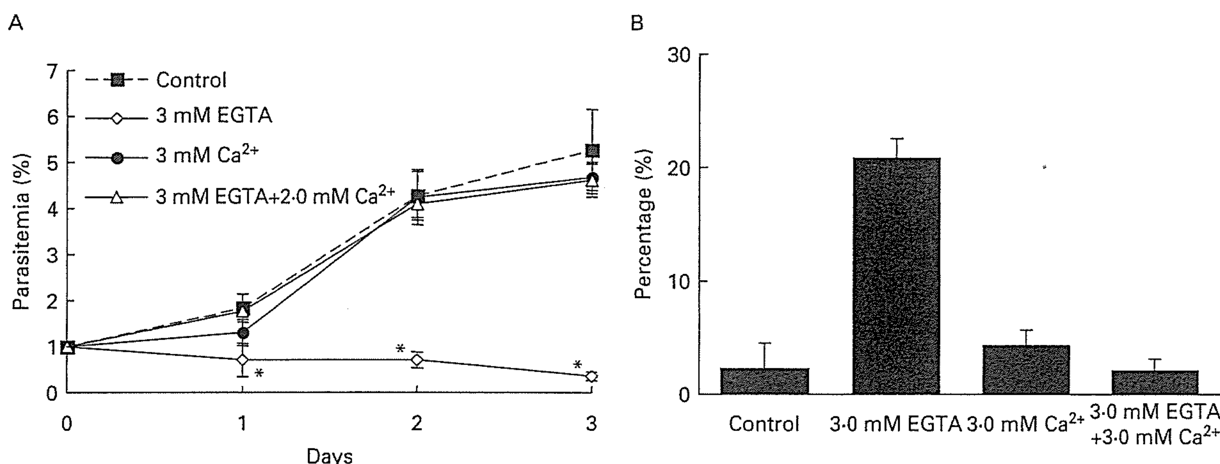

Fig. 4. Effect of addition of Ca^{2+} into the EGTA-treated *Babesia equi* culture on the parasite growth (A) and percentage of extracellular merozoites on day 1 of cultivation (B). Each value represents the mean ± S.D. in 3 separate wells. The asterisk indicates significant differences ($P<0{\cdot}05$) between the chemical-treated and control groups.

Although the growth of *B. equi* was completely inhibited in the presence of 3 mM EGTA, the culture combined with 3 mM Ca^{2+} showed a normal growth pattern (Fig. 4A) and normal percentage of extracellular merozoites (Fig. 4B) even in the presence of 3 mM EGTA. The percentage in the culture containing 3 mM Ca^{2+} without EGTA showed almost the same value as that of the control culture (Fig. 4B). Morphological changes were not observed in the EGTA-treated parasites.

Inhibitory effect of EGTA on the erythrocyte invasion by B. caballi

In the *in vitro* invasion test with *B. caballi*, erythrocyte invasion was significantly inhibited in the presence of EGTA at concentrations of 1·5 and 2 mM ($P<0{\cdot}05$) as compared to control invasion (Fig. 5). In contrast, a concentration of 1 mM EGTA did not show significant inhibition.

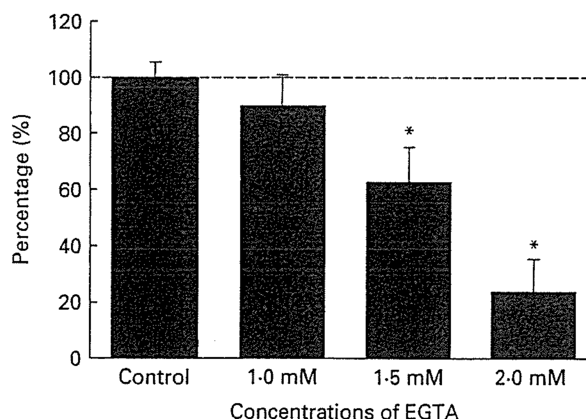

Fig. 5. Inhibitory effect of EGTA on the erythrocyte invasion by *Babesia caballi*. Relative values are expressed as the percentage of parasitaemia in the culture with EGTA to that with normal medium control (100%). Each value represents the mean ± S.D. in 3 separate trials. The asterisk indicates significant differences ($P<0{\cdot}05$) between the chemical-treated and control groups.

DISCUSSION

In the present study we demonstrated that supplementation of EGTA into the culture medium inhibited the *in vitro* growth of *B. caballi* and *B. equi* and that further addition of Ca^{2+} to the cultures negated the inhibitory effect of EGTA. These results indicate that EGTA has an inhibitory effect on the asexual growth of *B. caballi* and *B. equi*. Moreover, we observed abnormally increased numbers of extracellular merozoites in the cultures treated with high concentrations of EGTA in both parasites. These findings encouraged us to examine the possible consequences of inhibiting erythrocyte invasion by the parasites. Thus, an erythrocyte invasion test was carried out according to a recently established procedure (Franssen *et al.* 2003). For *B. caballi*, we confirmed that the EGTA had an inhibitory effect on erythrocyte invasion by the parasite. Therefore, we concluded that the level of Ca^{2+} is critical for erythrocyte invasion of *B. caballi* merozoites. We could not confirm whether Ca^{2+} plays an important role in the invasion of *B. equi* into erythrocytes using the same assay as for *B. caballi* since, despite several trials, we failed to collect undamaged free merozoites. Although the possibility of a harmful effect on the intracellular development of *B. equi* could not be completely excluded, we speculate that Ca^{2+} might also be important for the invasion of *B. equi* since an increase in extracellular parasites was found and no morphological changes were observed in the intracellular parasites of the EGTA-treated *B. equi* culture. Further study on the precise role that Ca^{2+} plays in the invasion of the *B. equi* parasite will be required in the future.

B. caballi was more sensitive to EGTA than *B. equi*. The IC_{50} values for *B. caballi* and *B. equi* were 1·27 mM and 2·25 mM, respectively. This might be due to the different components of RPMI 1640 and M199 used for *B. caballi* and *B. equi*, respectively. According to the data sheets (Sigma-Aldrich), M199 contains a higher concentration of Ca^{2+} (about 1·8 mM) than RPMI 1640 (about 0·5 mM). To completely chelate Ca^{2+}, the *B. equi* medium (M199) required a higher concentration of EGTA than the *B. caballi* (RPMI 1640) medium.

Several possible mechanisms for the role of Ca^{2+} in the erythrocyte invasion by *B. caballi* and, possibly, *B. equi* can be considered. The first possibility is that Ca^{2+} might be required for the specific molecular interaction between the protozoan ligands and the corresponding erythrocyte receptors that are essential for parasite invasion. In *B. bovis*, Ca^{2+} was reported to accentuate the binding affinity of the rhoptry-associated protein-1 (RAP-1) against bovine RBC (Yokoyama *et al.* 2002). The second possibility is that Ca^{2+} might play an important role in the vital signalling for the secretion of invasion-related molecules from the rhoptry or microneme organelle of *Babesia* parasites. Ca^{2+} is well established as a cellular messenger in many eukaryotic signal-transducing pathways (Moreno and DoCampo, 2003). In these pathways, Ca^{2+} functionally binds to the target proteins that play various roles as a second messenger in many biological reactions (Caroppo *et al.* 2004). In *T. gondii*, the secretion of a protozoan protein that relates to host cell invasion requires the release of Ca^{2+} from intracellular stores and the influx of Ca^{2+} from the extracellular milieu (Wetzel *et al.* 2004). Further study will be required to identify the relationship between erythrocyte invasion by the equine *Babesia* parasite and extracellular Ca^{2+}.

In conclusion, extracellular Ca^{2+} is suggested to be involved in erythrocyte invasion by equine *Babesia* parasites in the present study. These findings provide a deeper understanding of the invasion mechanism of equine *Babesia* into erythrocytes and could make a significant contribution to the development of culture systems for the discovery of anti-babesial drugs in the future.

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Immunochromatographic Test for Simultaneous Serodiagnosis of *Babesia caballi* and *B. equi* Infections in Horses

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An immunochromatographic test for the simultaneous detection of *Babesia caballi*- and *B. equi*-specific antibodies (BceICT) was developed using a recombinant *B. caballi* 48-kDa rhoptry protein (rBc48) and a recombinant truncated *B. equi* merozoite antigen 2 (rEMA-2t). An evaluation of the ability of the BceICT to detect antibodies in sera from uninfected horses and experimentally infected horses showed high sensitivities and specificities of 83.3% (10/12 sera) and 92.9% (52/56 sera), respectively, for the anti-*B. caballi* antibody and 94.1% (16/17 sera) and 88.2% (45/51 sera), respectively, for the anti-*B. equi* antibody. Results from the detection of antibodies in field-collected sera indicated that the BceICT results corresponded with those of enzyme-linked immunosorbent assays (ELISA), showing 91.8% correspondence (67/73 sera) for *B. caballi* and 95.9% correspondence (70/73 sera) for *B. equi*, and that the BceICT results also corresponded with the ICT for *B. caballi* and for *B. equi*, both of which were 98.2% (55/56 sera). The comparable results of the ICT and ELISA and the simplicity and rapidity of the performance of the ICT suggest that the BceICT would be a feasible test for the simultaneous serodiagnosis of both agents of equine babesiosis in the field.

Equine piroplasmosis, caused by *Babesia caballi* and *Babesia equi*, is an important protozoan disease worldwide from both veterinary and economic viewpoints (2). Various serodiagnostic tests have been developed for the disease, such as the complement fixation test (1, 11, 12), the indirect immunofluorescent antibody test (1, 11, 12), the enzyme-linked immunosorbent assay (ELISA) (1, 3, 5, 7, 8, 9, 10, 13, 14), the competitive-inhibition ELISA (9), and the immunochromatographic test (ICT) (6). In our previous studies, ELISAs for the serodiagnoses of *B. caballi* and *B. equi* infections demonstrated many advantages, such as higher sensitivity and specificity, lower cost of materials, and greater objectivity in the determination of results (5, 8), over the complement fixation test, indirect immunofluorescent antibody test, and competitive-inhibition ELISA. Compared with ELISA, however, the ICT is relatively simple, can be performed quickly, and has the listed advantages of ELISA (6).

Babesia caballi and *B. equi* have overlapping geographical distributions (4). In such areas, an individual horse may be infected by both species. Therefore, a test capable of detecting the antibodies induced by both types of parasites would be desirable. Here, we report an ICT for the simultaneous detection of *B. caballi*- and *B. equi*-specific antibodies (BceICT) that uses the recombinant *B. caballi* 48-kDa rhoptry protein (rBc48) and the recombinant truncated *B. equi* merozoite antigen 2 (rEMA-2t) as antigens for the simultaneous serodiagnosis of infections caused by two *Babesia* spp. in horses.

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MATERIALS AND METHODS

rEMA-2t. rEMA-2 was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase, as described previously (5). The fusion protein was purified using glutathione Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). The leader protein, glutathione *S*-transferase, was cleaved by thrombin protease.

rBc48. rBc48 was prepared as described previously, with some modification (7, 8). Briefly, the Bc48 gene inserted into pBluescript SK(+) vectors was subcloned into pGEX-4T (Amersham) of the bacterial expression vector after digestion with EcoRI and XhoI. The *E. coli* (BL21 strain) colony transformed with pGEX-4T/Bc48 was cultured on a small scale overnight in Luria-Bertani (LB) medium (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, and 0.1% 5 N NaOH) with 50 μg/ml of ampicillin sodium at 37°C. The overnight culture was then diluted to 1:100 in an LB medium for a large-scale culture at 25°C. When the optical density at 600 nm (OD_{600}) reached 0.50, *E. coli* was induced to express the rBc48 protein by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside and incubation for another 4 h at 25°C. The purification procedure for rBc48 was the same as that for rEMA-2t.

Conjugates. After dialysis in a 5 mM phosphate buffer at the proper pH (6.5 for rEMA-2t and 8.0 for rBc48), rEMA-2t and rBc48 were diluted to their optimal concentrations, 200 μg/ml and 125 μg/ml, respectively, and mixed gently with gold colloid particles (British BioCell International, SDX, United Kingdom) at the optimal pH. The ratio of volumes was 1:10. The mixtures were incubated at room temperature for 10 min without disturbance. Then, 0.05% polyethylene glycol 20,000 (PEG) and 1% bovine serum albumin (BSA) were added to stabilize and block the conjugate particles. After centrifugation at 18,000 × *g* for 20 min, 90% of the supernatants were discarded, and the pellets were resuspended in the remaining supernatants by sonication and then washed with phosphate-buffered saline containing 0.5% BSA and 0.05% PEG. Following the second centrifugation, the pellets were resuspended in phosphate-buffered saline with 0.5% BSA and 0.05% PEG until the OD_{520} reached 5. After the two conjugates were mixed and diluted in 10 mM Tris-HCl (pH 8.2) with 5% sucrose, the mixture was sprayed onto glass fiber (Schleicher & Schuell, NH) and dried in a vacuum overnight.

Rabbit anti-rEMA-2t IgG. A rabbit was immunized with 1 ml of rEMA-2t (2 mg/ml) mixed with 1 ml of complete Freund's adjuvant (Difco, Detroit, MI) by multiple intradermal injections into its dorsum. Two booster injections were given in a 2-week interval, with the same dose of antigen mixed with incomplete Freund's adjuvant (Difco). The rabbit was bled 10 days after the last booster. The immunoglobulin G (IgG) fraction was purified from blood serum with an Econo-

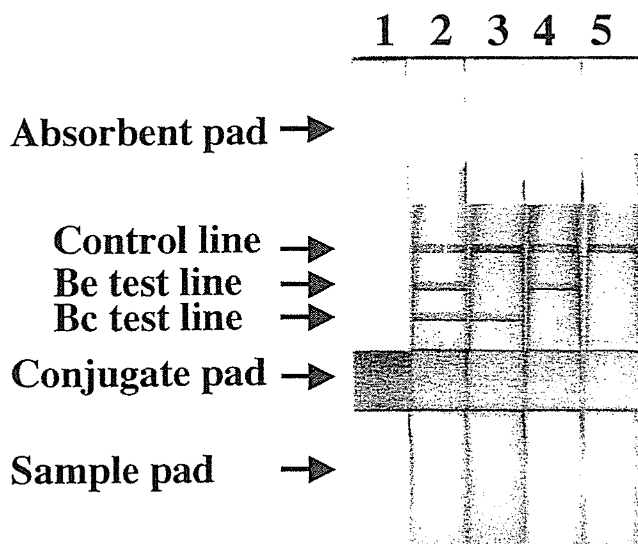

FIG. 1. Pretest (lane 1) and posttests (lanes 2 to 5) of BceICT strips. Bc test line: rBc48 was immobilized on the nitrocellulose membrane for the detection of antibody to *B. caballi*. Be test line: rEMA-2t was immobilized on the nitrocellulose membrane for the detection of antibody to *B. equi*. Lanes: 1, pretest; 2, antibodies to both *B. caballi* and *B. equi* are positive; 3, only the antibody to *B. caballi* is positive; 4, only the antibody to *B. equi* is positive; 5, the antibodies to both *B. caballi* and *B. equi* are negative.

Pac protein A kit (Bio-Rad, CA) according to the manufacturer's instructions and used as the control for the ICT.

Immobilization of rEMA-2t, rBc48, and rabbit anti-rEMA-2t IgG on nitrocellulose (NC) membrane. rEMA-2t (500 μg/ml), rBc48 (125 μg/ml), and rabbit anti-rEMA-2t IgG (1,500 μg/ml) were linearly jetted onto an NC membrane with a plastic backing (Schleicher & Schuell, NH) using a BioDot Biojet 3050 quanti-dispenser (BioDot, Inc., CA). The positions of the three lines are shown in Fig. 1. The membrane was dried at 50°C for 30 min and blocked with 0.5% casein in a 50 mM boric acid buffer (pH 8.5) for 30 min. After a wash with 50 mM Tris-HCl (pH 7.4) containing 0.5% sucrose and 0.05% sodium cholate, the membrane was air dried overnight.

Assemblage of test strips and detection of specific antibodies in horse serum. The NC membrane containing antigens and antibodies was assembled on an adhesive card (Schleicher & Schuell) with other components, such as an absorbent pad, a conjugate pad, and a sample pad, and cut into 6-mm-wide strips using a BioDot cutter (BioDot, Inc.), as shown in Fig. 1 (lane 1). Detection was performed by pipetting 100 μl of serum onto the sample pad. In the preliminary test, color in the control line took a maximum of 7 min to develop; color in the test lanes took a maximum of 15 min to develop, and the results did not change when the sample pad was read later than 15 min. Therefore, results were determined 15 min after the application of serum samples and recorded as (i) positive for both equine babesiosis species (Fig. 1, lane 2); (ii) positive for *B. caballi* and negative for *B. equi* (Fig. 1, lane 3); (iii) negative for *B. caballi* and positive for *B. equi* (Fig. 1, lane 4); and (iv) negative for both *B. caballi* and *B. equi* (Fig. 1, lane 5).

Sera. Thirty-nine uninfected sera were from race horses in Japan, a country assumed to be free of equine babesiosis. Twelve *B. caballi*- and 17 *B. equi*-infected sera were from horses infected experimentally with the parasites. Of the 73 field-collected sera, 56 were from horses in Jilin province, China, and 17 were from horses imported to Japan from different countries suspected of harboring *Babesia* infections.

RESULTS

Detection of specific antibodies against *B. caballi* and *B. equi* in sera from experimentally infected horses. The results of experiments for the detection of specific antibodies are summarized in Tables 1 and 2. The sensitivities and specificities of the BceICT were 83.3% (10/12 sera) and 92.9% (52/56 sera), respectively, for the detection of the antibody against *B. caballi* and 94.1% (16/17 sera) and 88.2% (45/51 sera), respectively, for the detection of the antibody against *B. equi* infection. The sensitivity of the BceICT for detecting antibodies to *B. caballi* (83.3%) and *B. equi* (94.1%) were equal to those of *B. caballi* ELISA (BcELISA) and *B. equi* ELISA (BeELISA). On the other hand, the specificity of the BceICT for detecting antibodies to *B. caballi* (92.9%) and *B. equi* (88.2%) were slightly lower than those of BcELISA (100%) and BeELISA (100%).

Detection of specific antibodies against *B. caballi* and *B. equi* in sera from horses in an area of endemicity. Comparisons between the BceICT and ELISAs in the detection of field-collected sera are shown in the last columns of Tables 1 and 2. The comparisons between the BceICT and BcICT and between the BceICT and BeICT are summarized in Table 3. The corresponding results were 91.8% (67/73) between BceICT and BcELISA, 95.9% (70/73) between BceICT and BeELISA, 98.2% (55/56) between BceICT and BcICT, and 98.2% (55/56) between BceICT and BeICT.

TABLE 1. Comparison of BceICT with BcELISA in the detection of specific antibodies against *B. caballi* in equine sera[a]

BcELISA result	No. of sera with indicated BceICT result from:							
	Uninfected sera (*n* = 39)		*B. equi*-infected sera (*n* = 17)		*B. caballi*-infected sera (*n* = 12)		Field sera (*n* = 73)	
	+	–	+	–	+	–	+	–
+	0	0	0	0	10	0	19	5
–	1	38	3	14	0	2	1	48
Total	1	38	3	14	10	2	20	53

[a] The sensitivity of both BcELISA and BceICT for detecting antibody to *B. caballi* was 83.3% (10/12), and the specificities of BcELISA and BceICT were 100% (56/56) and 92.9% (52/56), respectively.

DISCUSSION

The ICT is a nitrocellulose membrane-based immunoassay that relies on the migration of a liquid across the surface of the membrane by the capillary mechanism and the capture of the antibodies in the sample using the antigens in the mobile

TABLE 2. Comparison of BceICT with BeELISA in the detection of specific antibodies against *B. equi* in equine sera[a]

BeELISA result	No. of sera with indicated BceICT result from:							
	Uninfected sera (*n* = 39)		*B. equi*-infected sera (*n* = 17)		*B. caballi*-infected sera (*n* = 12)		Field sera (*n* = 73)	
	+	–	+	–	+	–	+	–
+	0	0	16	0	0	0	39	1
–	2	37	0	1	4	8	2	31
Total	2	37	16	1	4	8	41	32

[a] The sensitivity of both BeELISA and BceICT for detecting antibody to *B. equi* was 94.1% (16/17), and the specificities of BeELISA and BceICT were 100% (51/51) and 88.2% (45/51), respectively.