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Babesia divergens and *Plasmodium falciparum* Use Common Receptors, Glycophorins A and B, To Invade the Human Red Blood Cell

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Babesiosis has long been recognized as an economically important disease of cattle, but only in the last 30 years has *Babesia* been recognized as an important pathogen in humans. Invasion of erythrocytes is an integral part of the *Babesia* life cycle. However, very little information is available on the molecules involved in this process, in contrast to another hemoparasite, *Plasmodium falciparum*. Using invasion assays into normal red blood cells (RBCs), enzyme-treated cells, and clinically mutant cells, we showed that *Babesia divergens* uses neuraminidase- and trypsin-sensitive receptors to enter the RBCs, of which glycophorins A and B are the prominent ones. These results could have broad implications relating to evolutionarily conserved mechanisms of host cell entry in these related Apicomplexan parasites and pave the way toward a detailed molecular analysis of erythrocyte invasion in *B. divergens*.

Babesiosis, caused by infection with intraerythrocytic parasites of the genus *Babesia*, is one of the most common infections of free-living animals worldwide and is gaining increasing interest as an emerging zoonosis, a disease that is communicable from animals to humans (17, 26). The rodent parasite *Babesia microti* and the bovine pathogen *Babesia divergens* are responsible for most of the ~500 human infections that have been reported so far (26). Parasites that live in red blood cells (RBCs) have rather ingenious ways of gaining entry into these cells, thus escaping the dangers of the host immune system. Although there are many erythrocyte-seeking protozoa, most data regarding erythrocyte invasion have come from studies on *Plasmodium*, a related Apicomplexan parasite. In both parasites, invasion has been shown to be composed of an initial phase of random cell-cell contact, subsequent reorientation, and specific receptor-ligand interactions (6, 14, 15, 25). However, in contrast to *Plasmodium falciparum*, little is known about the molecules used by *Babesia* to attach and invade erythrocytes, and yet this is one of the most critical factors in the life cycle of the parasite.

The *Babesia* blood stage merozoite is designed for one major role: to locate, bind to, and invade host RBCs. This is a very specific interaction; *Babesia* does not invade other host cells (15, 26). This specificity implies the presence of a receptor(s) on the erythrocyte, which is recognized by a complementary ligand(s) on the parasite. Several *Babesia* molecules have been shown to bind RBCs in vitro and may thus play a role in invasion. In *Babesia bovis*, a merozoite surface antigen (MSA-2) (21) and a rhoptry-associated antigen (RAP-1) (22) have been shown to bind erythrocytes. In *Babesia equi*, two other merozoite antigens, EMA-1 and EMA-2, have also been shown to interact with the RBC membrane skeleton (18). The cognate RBC receptors for these molecules remain unknown, as has the identity of any erythrocytic receptor used by the invading *Babesia* merozoite.

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The work presented in this report is based on the hypothesis that erythrocyte invasion by *B. divergens* and *P. falciparum* may be conserved in terms of the molecular machinery used. For malaria, a combination of biochemical and biological studies involving enzymatic modification of the RBC surface membranes and the use of RBC variants have clearly shown differences in the susceptibility of various RBCs to malaria invasion and have indicated some essential receptor-ligand interactions (2, 4, 13, 20, 23, 28). Through such studies, all three major glycophorins (GPA, GPB, and GPC) and band 3 have been implicated in malaria invasion. We have used similar strategies to look at molecules that are critical for invasion in *B. divergens* and have found that GPA and GPB also play a role in the entry of the *B. divergens* merozoites, as invasion decreases substantially in cells lacking either receptor. This is the first identification of any host cell receptor for any of the *Babesia* species.

The assay of choice to validate molecules that participate in invasion is an inhibition-of-invasion assay, in which changes in parasitemia are assessed in an invasion assay relative to a wild-type assay. Inhibition of invasion can be achieved by modification of different components of the assay or by the addition of competitors of the molecules that participate in invasion. Similar assays have laid the foundation of all invasion work done with *Plasmodium* (3, 12, 19, 24, 27). The first clue, from our studies and others (29), that participation of the sialic acid-bearing receptors was necessary for *B. divergens* invasion came from using enzyme-treated RBCs in invasion assays (Fig. 1). Specific moieties of different surface molecules are cleaved by the judicious use of enzymes like neuraminidase, trypsin, and chymotrypsin. Erythocytes were washed three times in RPMI 1640 before treatment with neuraminidase, trypsin, or chymotrypsin. Briefly, 0.1 ml of packed RBCs was treated with either 1 ml of a 19.4-mg/ml concentration of trypsin (TPCK) (Sigma), 1 ml ofa 1-mg/ml concentration of chymotrypsin (Sigma), or 0.1 ml of a 0.1-IU/ml concentration of neuraminidase (Sigma) for 30 min at 37°C. The RBCs were washed extensively in phosphate-buffered saline and RPMI 1640 before use in invasion. The *B. divergens* Rouen 87 isolate was propagated in an in vitro culture as described previously (11).

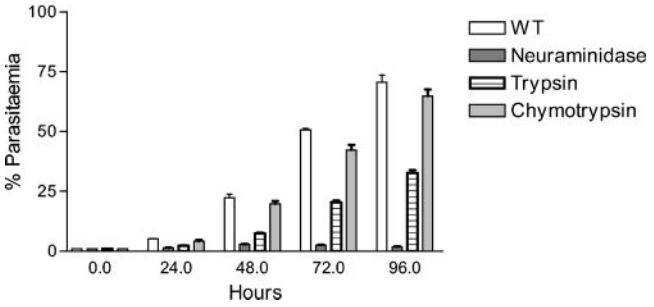

FIG. 1. Inhibition of invasion of *B. divergens* into enzyme-treated RBCs compared to untreated (WT) RBCs. Parasitemia (parasite-infected RBCs per100 RBCs counted) was assessed over a period of 4 days. A minimum of 1,000 cells were counted for each data point. Each bar indicates the average value for three individual experiments, and the error bars indicate standard deviations.

The invasion of *B. divergens* into the enzyme-treated cells, relative to invasion in untreated cells, was monitored over 4 days. Aliquots of cultures were withdrawn every 24 h for 4 days, and parasitemia was assessed by Giemsa staining. The 90% inhibition of invasion that we obtained in neuraminidase-treated RBCs (Fig. 1), relative to wild-type RBCs, pointed to a significant role for the glycophorins (GPA, GPB, GPC, and GPD), as they are the major sialylated proteins on the RBCs. Results from this assay also pointed specifically to the participation of trypsin-sensitive molecules in invasion (Fig. 1), with ~45% reduction in parasitemia in trypsin-treated RBCs. A similar reduction in invasion has also been obtained with other *Babesia* species, including *B. bigemina* and *B. bovis* (9, 15, 16). While we recognize the fact that enzyme treatment is relatively broad in its scope and results in modifications to more than one molecule, we used this assay as a first line of investigation.

We next used rare, clinical mutant RBCs as host cells in the invasion assay. These RBCs possess genetically determined defects which are usually more discrete and well defined than defects produced by treatment with proteolytic enzymes and thus are better suited to yield specific molecular information. The mutant RBCs were obtained from the rare-RBC repository at the New York Blood Center. Results from assays of invasion into cells lacking four different surface molecules, monitored over 4 days, are shown in Fig. 2. We obtained a drastic reduction in invasion (45%) into cells lacking GPA (Fig. 2A)and a more moderate but significant (25%) inhibition of invasion in cells lacking GPB (Fig. 2B). These results have clearly identified GPA and GPB as putative receptors for *B. divergens*. In contrast, the Duffy (Fig. 2C) and Kell blood group antigens (Fig. 2D) were not involved in the invasion process, as cells deficient in these antigens, as well as wild-type RBCs, are susceptible to invasion. Residual invasion in enzyme-treated and mutant RBCs point to additional pathways (apart from GPA and GPB) of invasion for the *B. divergens* merozoite.

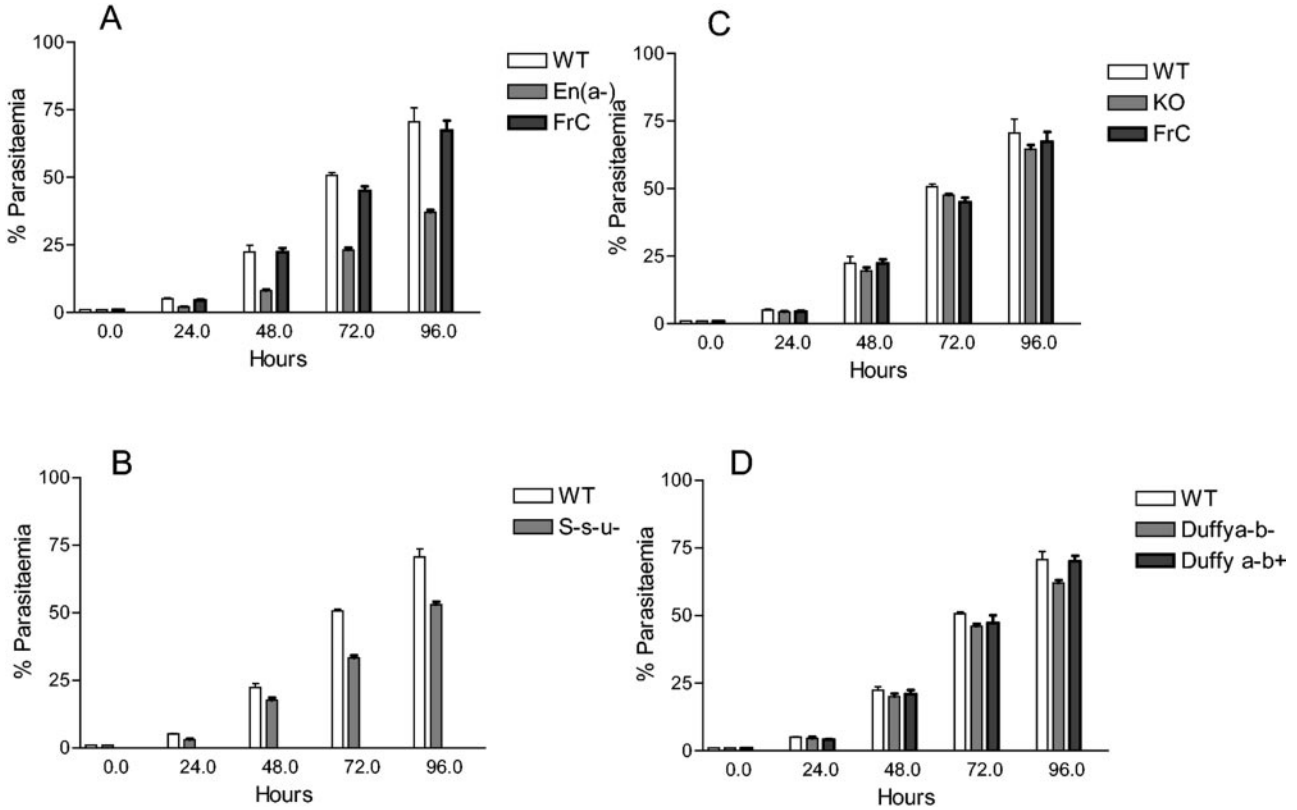

FIG. 2. Inhibition of invasion of *B. divergens* into mutant RBCs: cells lacking GPA [En(a-)] (A), cells lacking GPB (S-s-u-) (B), Kell null cells (KO) (C), and Duffy mutants (D). Frozen control RBCs (FrC) which were age- and storage-matched cells for the En(a-) and Kell null cells were included in the experiments illustrated in panels A and C. Samples were obtained from at least two different donors. Each data point represents the average value for three individual experiments, and the error bars indicate standard deviation.

Thus, as in *P. falciparum*, multiple pathways of invasion may exist to ensure successful completion of this critical step in the *B. divergens* life cycle.

These results highlight the similarity of invasion pathways utilized by these two hemoparasites and thus lay the foundation for a systematic evaluation of the erythrocytic receptors and the parasite ligands that have been shown to be employed by *P. falciparum* which may also function similarly in *B. divergens*. We are currently looking for molecules in *B. divergens* that are homologous to the erythrocyte binding ligand (ebl) (1) and the reticulocyte binding protein (RBP) families (5, 10) of *P. falciparum*. Of interest in this context are the recent reports of molecules homologous to PfAMA-1 and PfTRAP that have been identified in *B. bovis* (7, 8). This report substantiates our hypothesis on the conservation of the molecular machinery involved in invasion by these two Apicomplexan parasites into the same host cell, the human erythrocyte, and thus paves the way for a comparative analysis of these molecules in the two parasites.

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