Babesia bovis: Subcellular localization of host erythrocyte membrane components during their asexual growth

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Abstract

In the present study, the subcellular localization of the host red blood cell (RBC) membrane components, the α2-3-linked sialic acid (SA) residues and the lipid bilayer, was observed during the asexual growth of *Babesia bovis* using two erythrocyte probes, the SA-specific lectin (MALII) and the lipophilic fluorescent (PKH2) probes, respectively. In confocal laser scanning microscopy with MALII, the SA residues on the surface of parasitized RBCs appeared to accumulate into the intracellular parasites as the parasites matured as well as to remain on the surface of extracellular parasites. Furthermore, when PKH2-labeled RBCs were infected with *B. bovis*, PKH2 signals were also observed around both the intracellular and the extracellular parasites, similarly to the results of MALII. These results indicated that the components derived from the host erythrocyte membrane are incorporated into the intracellular parasites during their asexual growth within the parasitized RBC, suggesting the possible formation of a parasitophorous vacuole-based network or a parasite surface coat.

Index descriptors and abbreviations: *Babesia bovis* (*B. bovis*); SA, sialic acid; MALII, *Maackia amurensis* lectin II; RBC, red blood cell; RT, room temperature; PBS, phosphate-buffered saline; *Babesia equi* (*B. equi*); PV, parasitophorous vacuole.

Babesia bovis, a hemoprotozoan parasite that grows asexually within bovine red blood cells (RBCs), causes a virulent disease in cattle characterized by fever, anemia, hypotensive shock syndrome, and, in severe cases, a fetal cerebral attack (Homer et al., 2000). The *B. bovis* infection, called bovine babesiosis, often results in great economic losses in the livestock industry in many tropical and subtropical regions of the world (de Waal and Combrink, 2006). In the case of *Babesia* parasites, various associations with the host RBCs are required for their invasion process, the subsequent maintenance of their intracellular existence, and the elicitation of pathogenesis (Carcy et al., 2006, de Vries et al., 2006, Yokoyama et al., 2006).

Recently, sialic acid (SA), which is the terminal or subterminal non-reducing unit attached to the underlying galactose residues of many sialoglycoproteins on the host cell membrane (Varki, 2001), has been shown to play an important role in the RBC infection by *Babesia* parasites (Gaffar et al., 2003, Kania et al., 1995, Lobo, 2005, Yokoyama et al., 2006, Zintl et al., 2002). Gaffar et al. (2003) reported that the SA residues are important for the invasion process of *B. bovis* into the host RBCs. In addition, our recent study suggested that the α2-3-linked SA residues on host RBCs are essential not only for invasion process by *Babesia caballi* but also for intraerythrocytic maturation of *Babesia equi* (Okamura et al., 2005). However, the manner in which the *Babesia* parasites utilize the host SA for their infection and growth has not been elucidated. To further investigate the association of *B. bovis* with the host RBC membrane during their asexual growth, the localization of the SA residues was examined by confocal laser scanning microscopy on SA-specific lectin-stained smears

from an *in vitro* culture of the parasite. In addition, fluorescence-tagged fresh RBCs were added into the parasite culture, and the fate of the fluorescent membrane was observed in the newly parasitized RBCs in relation to the localization of the SA residues.

In the present study, the Texas strain of *B. bovis* was cultured in a GIT medium (Nihon Pharmaceutical, Tokyo, Japan) according to a previous report (Bork et al., 2005). Thin blood smears were fixed on glass slides with 50% acetone-methanol at -30 ºC for 3 min; then, the slides were reacted with 10 µg/ml of the biotinylated *Maackia amurensis* lectin II (MALII; Vector Laboratories, Inc., Burlingame, CA, USA), which specifically labels the α2-3-linked SA residues (Knibbs et al., 1991, Okamura et al., 2005), and subsequently with 10 µg/ml streptavidin-Alexa Fluor® 488 conjugate (Molecular Probes, Eugene, OR, USA). In both cases, the reaction was conducted for 1 h at room temperature (RT) in 3% bovine serum albumin in phosphate-buffered saline (PBS). Finally, the slides were reacted with 25 µg/ml propidium iodide (Molecular Probes) containing 50 µg/ml RNase A (Roche Applied Science, Mannheim, Germany) for 30 min at RT so that the parasites' nuclei would be stained; the slides were then examined with a confocal laser scanning microscope equipped with a TCN-SC software (Leica Microsystems, Heidelberg, Germany) (Bork et al., 2004, Yokoyama et al., 2002). To provide a simultaneous demonstration of a negative control for MALII detection in identical smears, an excess volume of *Vibrio cholerae* neuraminidase (Sigma, St. Louis, MO, USA)-pretreated RBCs (Okamura et al., 2005) was mixed in the standard *Babesia* cultures with intact RBCs immediately before the preparation of the smears. This

preparation provides an easy differentiation between naturally sialylated RBCs (containing the MALII-positive unparasitized and parasitized RBCs) and artificially desialylated RBCs (serving as negative controls for MALII detection) in identical smears. The experiment was repeated 5 times, and the reproducibility of the results was confirmed. For an objective interpretation of the data, colorimetric profiles, such as the contrast and luminosity of photomicrographs, were standardized among experiments.

As a result, a characteristic localization of α2-3-linked SA residues was visualized in the parasitized RBCs using MALII, as shown in Figure 1A. The intensity of the α2-3-linked SA residues gradually decreased from the entire surface of the parasitized RBCs with the maturation of intracellular parasites (Fig. 1A, panels ii and iii) but remained all over the surface of normal RBCs (Fig. 1A, panel i). Simultaneously, the intracellular parasites acquired an intense reaction with MALII, which became stronger as the parasites matured (Fig. 1A, panels ii and iii). Furthermore, a clear reaction with MALII was also observed around the extracellular parasite (Fig. 1A, panel iv). In contrast, the MALII lectin could not recognize the desialylated RBCs that had been pretreated with *V. cholerae* neuraminidase (Fig. 1A, panel v).

In another experiment, the *B. bovis* culture was mixed with an excess volume of pre-labeled RBCs with a lipophilic green fluorescent probe (PKH2: Zynaxis, Inc., Malvern, PA, USA) (Horan et al., 1990) and then incubated. The culture was collected at 12 h post-initiation of the culture, washed 2 times in PBS, and then stained with 10 μM SYTO-64 (Molecular Probes), a cell-permeable red fluorescent nucleic acid dye (Zhang and Poo, 2002). After 3 washes in PBS, the culture was allowed to adhere on

glass slides pre-coated with poly-L-lysine (Sigma) by incubating for 30 min at RT. The slides were covered by glass slips without fixation of the cells and then examined with a confocal laser scanning microscope as described above. The experiment was repeated 5 times, and the reproducibility of the results was confirmed.

The growth efficiency of *B. bovis* with PKH2-labeled RBCs was the same as that of the control culture that had been mixed with unlabeled RBCs (data not shown), and the various stages of parasite maturation were observed at 12 h post-initiation of the culture, as shown in Figure 1B. Under the microscope, normal RBCs showed widespread defused fluorescence of PKH2 (Fig. 1B, panel i). When the labeled RBC became parasitized, the membrane-derived component of the RBC was definitely observed around the intracellular parasites as they matured (Fig. 1B, panels ii and iii). Similarly to the result of SA localization (Fig. 1A), the PKH2 signal was also observed around the extracellular parasite (Fig. 1B, panel iv). None of the parasitized RBCs and intracellular and extracellular parasites showed fluorescence at the beginning of this experiment (Fig. 1B, panel v).

In the present study, confocal laser microscopic analyses with the two probes, MALII and PKH2, indicated that intracellular *B. bovis* incorporates the host SA residues and the RBC lipid bilayer, respectively. A decreasing intensity of SA in the membrane of parasitized RBCs and an increasing intensity of SA in the intracellular parasites as the parasites matured were observed. Since the importance of SA residues in the RBC infection by *B. bovis* has been reported by other studies with a different strain of *B. bovis* (Gaffar et al., 2003) and other *Babesia* spp. (Kania et al., 1995, Zintl et al., 2002),

the incorporation of SA from the host RBC during intracellular maturation suggests that it is conserved in other *Babesia* strains. Importantly, the present data may support our previous finding that *B. equi* did not grow in parasitized RBCs lacking SA residues on the surface (Okamura et al., 2005).

In *Plasmodium falciparum*, the host cell membrane is invaginated during the parasite entry, resulting in the formation of a parasitophorous vacuole (PV) surrounding the intracellular parasite. Next, the parasite extends the PV to the host membrane by forming a tubovesicular membrane network and then develops antigenic and structural alterations in the cytoplasm and membrane of the parasitized RBC to gain pathways of nutrient import and parasite protein export (Haldar et al., 2002). The results of this study suggest the possibility of the formation of a PV membrane-based network by *B. bovis* within the parasitized RBC. *Babesia* parasites can escape from the PV into the host cell cytosol shortly after invasion (Moltmann et al., 1983, Rudzinska et al., 1976). However, the apparent formation of PV by *Babesia* spp. during their intracellular maturation has never been shown, not even in studies involving electron microscopic observations (Kawai et al., 1999, Potgieter and Els, 1977). An earlier study (Todorovic et al., 1981) showed that the body of *B. bovis* is covered with a peculiar complex consisting of two inner and one outer membrane inside the parasitized RBC. These combined results suggest that the outermost membrane might be derived from the host RBC membrane containing α2-3-linked SA residues. Otherwise, the host-derived SA on the parasite might have another role(s) after the egress of parasitized RBC, such as the immune evasion seen in *Trypanosoma cruzi*, which also incorporates the SA from the

mammalian host cells onto the parasite membrane by the parasite's trans-sialidase (Schenkman et al., 1993). Sialylation may permit the parasite to shield itself from antibody-induced lysis (Pereira-Chioccola et al., 2000) and induce anergy in immune-competent cells (Kierszenbaum et al., 1999). In further studies, it will be interesting to determine whether the transferred SA on the extracellular parasite facilitates the evasion from the host immune system for the extracellular survival of the parasite.

The interaction of *Babesia* parasites with SA residues in the host animals might also offer a potential role of the SA in the vector stage of *Babesia* parasites in the transmitted ticks, *Boophilus* spp. SA-specific lectin is found in the hemolymph of *Ixodes ricinus*, one of the main vectors of *Borrelia burgdorferi*, and has been suggested to mediate the bacterial adherence to the tick cells via the SA that is present in the bacterial cell wall (Kuhn et al., 1996). It is possible that *Babesia* parasites somehow obtain the SA from the host animals after entering the host's circulation system and/or from the tick during their gametogony/sporogony. Thus, a better understanding of the host-parasite interaction in babesiosis could accelerate further studies on the complex interaction of the host, vector, and pathogen and augment our knowledge on the subject.

Acknowledgments

We wish to thank the staff and students of the National Research Center for Protozoan Diseases for their critical comments and technical assistance. This research was supported by Grants-in-Aid for Scientific Research from the Japan Society for the

Promotion of Science, the Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, Grants from the Bio-oriented Technology Research Advancement Institution (BRAIN), and The 21st Century COE Program (A-1), Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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Figure captions

Fig. 1. Confocal laser scanning microscopy for *B. bovis*-parasitized RBC. (A) The localization of α2-3-linked SA residues was visualized by MALII staining. Representative photographs of unparasitized RBC (i) and ring-form (ii), pear-shape (iii), and extracellular (iv) parasites are shown. The top panels show the transmission micrographs, while the bottom panels show the fluorescence micrographs (α2-3-linked SA residues in green and parasites' nuclei in red). Panel v shows the lack of reaction of neuraminidase-treated RBC with MALII due to desialylation before the assay. Since these micrographs are from *B. bovis* cultures mixed with the neuraminidase-treated RBC, the surrounding RBC without fluorescence in the panels i-iv indicate the neuraminidase-treated RBC with no reaction with MALII. (B) The fate of the erythrocyte membrane lipid layer was traced during the growth of *B. bovis*. The RBC pre-labeled with PKH2 were mixed into the parasite culture, and *B. bovis*-parasitized RBC were photographed at 12 h post-initiation of the culture. Fluorescence micrographs (PKH2-labeled erythrocyte membrane in green and parasites' nuclei in red) of

unparasitized RBC (i) and ring-form (ii), pear-shape (iii), and extracellular (iv) parasites are representatively shown. Panel v shows the non-fluorescent parasitized RBC observed at the beginning of this experiment. The bars represent 5 μm.

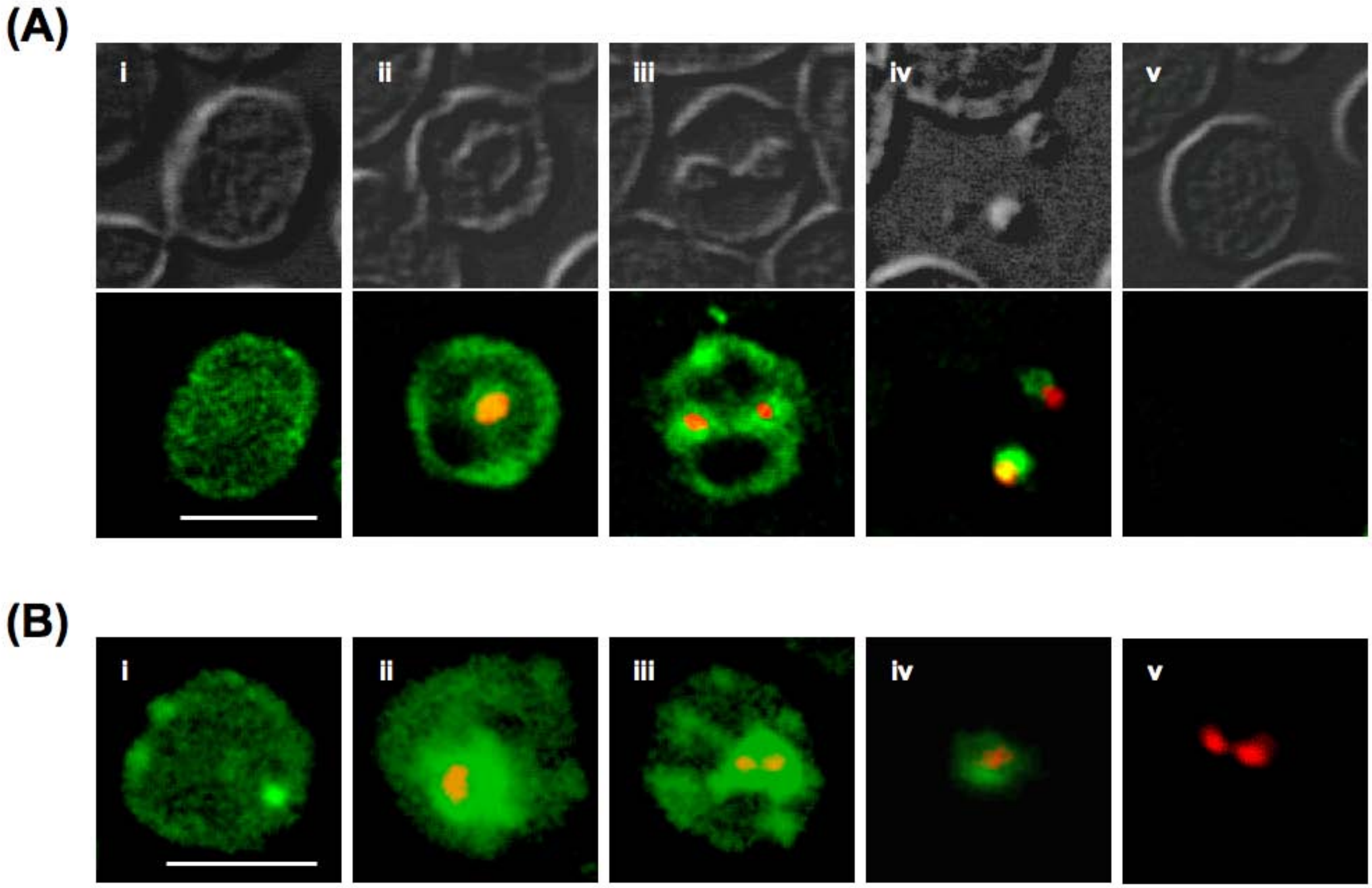

Fig. 1. Okamura et al.