FULL PAPER *Parasitology*

Involvement of a Host Erythrocyte Sialic Acid Content in *Babesia bovis* Infection

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ABSTRACT. Host sialic acid (SA) has recently been suggested to play an important role in erythrocyte (RBC) infection by *Babesia* spp. The present study attempted to further determine the specific type of SAs important in the RBC invasion. Bovine RBC was found to bear abundant α2–3-linked SA residues but not α2–6-linked SA in nature, confirmed by flow cytometric analysis of the neuraminidase (Nm)-treated RBCs. Lectin-blot analyses revealed the removal of α2–3-linked SAs from the 97-, 33-, and 31-kDa bands by the Nm treatment. Addition of the Nm-treated RBCs into an *in vitro* culture of *B. bovis* resulted in a decreased population of the parasitized RBCs. The thin smear samples from the cultures were then observed under a confocal laser scanning microscope after staining with the α2–3-linked SA-specific lectin: a selective invasion of *B. bovis* was found only in the intact RBCs bearing the SAs, but not in the desialylated RBCs. Furthermore, a significant reduction of the parasitized RBCs was also observed in the culture supplemented with exogenous 3'-sialyllactose containing the α2–3-linked SAs. However, the complete inhibition of parasite proliferation was not achieved in the culture. These findings indicate that while the α2–3-linked SA-dependent pathway is needed for highly efficient invasion of host RBCs by *B. bovis*, there might also be other potential alternative pathways.

KEY WORDS: *Babesia bovis*, erythrocyte, invasion, sialic acid.

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Babesia bovis, a hemoprotozoan parasite invading and replicating within bovine erythrocytes (red blood cells; RBCs), causes a serious disease characterized by fever, anemia, hypotensive shock syndrome, and, in severe cases, a fetal cerebral disease with a high mortality rate [9, 28]. Therefore, the spread of *B. bovis* infection results in great economic losses all over the world [2]. Much effort has so far been directed to the development of effective control strategies, including vaccination and chemotherapy against the bovine babesiosis [3, 26]. However, since detailed information about the molecular interactions between *Babesia* parasites and the host RBCs is limited, the control of babesiosis is still insufficient.

Recently, sialic acids (SAs), which are the terminal or subterminal non-reducing units attached to the underlying galactose residues of many sialoglycoproteins on the host cell membrane [24], have been shown to play an important role in RBC invasion by *Babesia* parasites [7, 12, 33]. Gaffar *et al.* [7] reported that the SA residues, regardless of the type of linkages (α2–3- and α2–6-linkages), are important in the RBC invasion by *B. bovis* and that *B. bovis* can invade not only bovine RBCs but also human and other animal RBCs in an SA-dependent manner. Interestingly, our recent study suggested that the host SAs are important not only for RBC invasion by *B. caballi* but also for the intraerythrocytic maturation of *B. equi* [16]. However, the knowledge of molecular interaction between *Babesia* parasites and host SAs is still limited. The present study, therefore, attempts to further investigate a potential involvement of α2–3-linked SAs in the *B. bovis* invasion into host RBCs. The elucidation of the exact role of host SAs in the RBC infection can facilitate the discovery of appropriate vaccine candidates and the development of novel drugs to inhibit the RBC invasion and/or the intraerythrocytic development of *Babesia* spp.

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MATERIALS AND METHODS

Parasite: The *B. bovis* Texas strain was cultured *in vitro* according to a microaerophilus stationary-phase culture method [15]. Briefly, the parasite was cultured with bovine RBCs at a 10% packed cell volume in a GIT medium (Nihon Pharmaceutical, Tokyo, Japan) [1, 10] with an antibiotic-antimycotic (100 ×) supplement (Invitrogen Corp., Carlsbad, CA, U.S.A.) in 24-well plates (Nalge Nunc International, Rochester, NY, U.S.A.) in 5% CO_2 and 5% O_2 at 37°C. RBCs were collected for the *in vitro* culture by centrifugation at 1,100 × *g* for 7 min at 4°C, after defibrination of blood retrieved from a selected healthy Holstein cow by shaking with glass beads for 30 min at room temperature (RT). The RBCs were then stored in a Vega y Martinez (VyM) buffer [25] at 4°C before use. The cultures were routinely maintained by changing the medium daily, and subcultures were made when the parasitemia (percentage of parasitized RBCs to a total of more than 1,000 RBCs) reached between 5–10%.

Neuraminidase (Nm) treatments of bovine RBCs: For the enzymatic treatment, two different neuraminidases (Nm) were employed: *Vibrio cholerae*-neuraminidase (Vc-Nm; Sigma, St. Louis, MO, U.S.A.) and *Arthrobacter ureafaciens*-neuraminidase (Au-Nm; Calbiochem, San Diego, CA, U.S.A.), which preferentially cleave α2–3- and α2–6-linked SA, respectively [5]. A 0.4-m*l* portion of freshly prepared bovine RBCs was separately treated with each Nm at various concentrations for 3 hr at 37°C on a rotator [16]. The treated RBCs were then thoroughly washed 5 times in cold phosphate-buffered saline (PBS) by centrifugation at 1,100 × *g* for 7 min at 4°C and suspended in a VyM buffer to store at 4°C before use. For the control, PBS-treated RBCs were prepared in the same manner as for the Nm treatment.

Flow cytometric analyses: *Maackia amurensis* lectin II (MAL II; Vector Laboratories, Inc., Burlingame, CA, U.S.A.) and *Sambucus nigra* lectin (SNL; Vector Laboratories, Inc.) were used to specifically label the α2–3- and α2–6-linked SAs, respectively, for the flow cytometric analyses. For lectin labeling, 5 *μl* of Vc-Nm-, Au-Nm-, and PBS-treated RBCs were reacted with biotinylated MAL II or SNL diluted at 10 *μ*g/m*l* in a washing buffer (5% bovine serum albumin (BSA) and 1 mM EDTA in PBS) on ice for 1 hr, followed by 3 washes in a washing buffer. The RBCs were then reacted with streptavidin-Alexa Fluor® 488 conjugate (Molecular Probes, Eugene, OR, U.S.A.) diluted at 10 *μ*g/m*l* in a washing buffer. The Vc-Nm-, Au-Nm-, and PBS-treated RBCs without any lectin labeling were used for the control study. After washing 3 times, the RBCs were resuspended in 4 m*l* of a washing buffer. All the reactions and washings were performed at 4°C. The lectin-labeled RBCs were analyzed using a flow cytometer (Epics XL™ with System II™ software, Beckman Coulter, Miami, FL, U.S.A.) in the optimal setting for bovine RBCs according to an earlier study [29]. Only the singly separated cells were gated; of them, 10,000 RBCs were analyzed. A reactivity of SNL to α2–6-linked SAs was confirmed using murine RBCs as the positive control.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and lectin blot analyses: The RBC ghosts were prepared by hypotonic lysis of Vc-Nm- or PBS-treated RBCs in a 10-mM phosphate buffer [6]. Two mg/m*l* of ghost membrane proteins was mixed with an equal volume of an SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.01% bromphenol blue), sonicated for 1 min, and then heated at 100°C for 5 min. The ghost samples (10 *μ*g/lane) were subjected to SDS-PAGE on a 10% polyacrylamide gel, followed by Coomasie Brilliant Blue (ICN Biochemicals Inc., Aurora, OH, U.S.A.) staining.

For lectin blot analysis, the ghost membrane fractions were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.) from the polyacrylamide gel and then immersed in 7% BSA-PBS overnight at 4°C. After 3 washes in PBS, 10 *μ*g/m*l* biotinylated MAL II was applied on the membrane strips. Bound lectin was subsequently reacted with 1 *μ*g/m*l* horseradish peroxidase-coupled streptavidin (Molecular Probes) and detected by an enhanced chemiluminescence using WesternLightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA, U.S.A.).

Effect of Nm-pretreated RBCs on the in vitro growth of B. bovis: *B. bovis*-infected RBCs, whose parasitemia had been adjusted to 5% with intact RBCs, were mixed with Nm-treated RBCs at 1% parasitemia in a GIT medium in 96-well tissue culture plates (Nalge Nunc International). This procedure resulted that 80% of the total RBCs in the cluture was Nm-treated ones [16]. PBS-treated RBCs were used for the control. The assay was carried out in 5 wells for each treatment in 3 separate experiments. At 24 hr post-initiation of the culture, parasitemia was determined and translated to the percent growth relative to the control parasitemia in the PBS-treated RBCs: relative growth (%)=(parasitemia in Nm-treated cells/parasitemia in PBS-treated cells) × 100. Statistical analyses were performed by one-way ANOVA with Dunnet's post-test (InStat®, GraphPad Software, San Diego, CA) for a comparison of the percent growth. Differences were considered significant when P values of less than 0.05 were obtained.

Thin blood smears obtained from the parasite cultures with Nm-treated RBCs as described above were also observed in confocal laser scanning microscopy to see how the host sialic acids are involved in the RBC infection by *B. bovis*. After fixation with 50% acetone-methanol at –30°C for 3 min, the smears were reacted with 10 *μ*g/m*l* of the biotinylated MAL II in 3% BSA in PBS after blocking with 3% BSA in PBS. The slides were washed 3 times in PBS and then reacted with 10 *μ*g/m*l* streptavidin-Alexa Fluor® 488 conjugate for 1 hr at RT. Following 3 washes in PBS, they were reacted with 25 *μ*g/m*l* propidium iodide (PI) (Molecular Probes) containing 50 *μ*g/m*l* RNase A (Roche Applied Science, Mannheim, Germany) for 30 min at RT to stain the parasites' nuclei [32]. The slides were washed 3 times in PBS, covered with glass slips using 50% glycerol-PBS, and then examined with a confocal laser scanning microscope equipped with TCN-SC software (Leica Microsystems, Heidelberg, Germany).

In vitro growth inhibitory effects of exogenous sialic acids and sialyllactoses: For the *in vitro* growth inhibition assays, *N*-acetylneuraminic and *N*-glycolylneuraminic acids (Neu5Ac and Neu5Gc, respectively; Dextra Laboratories, Reading, UK) and 3'- and 6'-sialyllactoses (Glycotech, Gaithersburg, MD, U.S.A.) were added to a GIT medium with 50 mM HEPES (Invitrogen Corp.) at 1.0 mM for the culture of *B. bovis* (1% parasitemia) [16]. For the control study, a medium without any compounds or with lactose (Sigma) was prepared. The data were obtained from smear samples taken at 24 hr post-initiation of the culture and statistically analyzed as described above.

RESULTS

Effect of Nm treatment on the bovine RBC membrane: MAL II and SNL are known to selectively recognize α2–3-

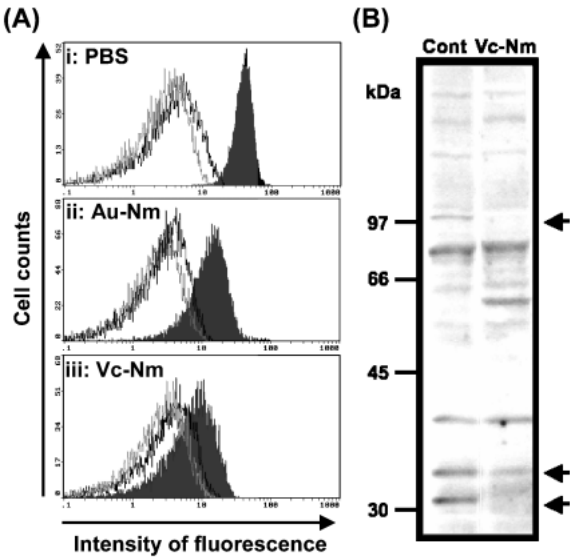

Fig. 1. (A) Flow cytometric analyses of PBS-treated (i), 200 mU/m*l* Au-Nm-treated (ii), and Vc-Nm-treated (iii) RBCs reacted with *Maackia amurensis* lectin MAL II (shaded area) or *Sambucus nigra* lectin SNL (black line) or without any lectin reaction (gray line). (B) Lectin blot analysis of the membrane ghosts with MAL II. Molecular weight standards are indicated in kDa on the left. Vc-Nm treatment led to the disappearance of the 97-, 33-, and 31-kDa MAL II-specific bands, as indicated by arrows on the right, and the appearance of a 58.5-kDa band.

linked and α2–6-linked SAs, respectively [14, 20, 21, 27]. In flow cytometric analyses, SNL showed an undetectable reaction with PBS-treated RBCs, but reacted well to the murine RBCs (data not shown). In contrast, a high-intensity binding of MAL II to PBS-treated RBCs was observed, indicating that bovine RBCs bear a high amount of α2–3-linked SAs but not of α2–6-linked SAs (Fig. 1A, i). Significantly, a reduced binding of MAL II to the 200 mU/m*l* Vc-Nm-treated RBCs was detected, but there was no difference in the SNL binding between the PBS- and Vc-Nm-treated RBCs (Fig. 1A, iii). The Au-Nm-treated RBCs also showed no decrease in the binding of SNL, even when the RBCs were treated with the highest concentration (200 mU/m*l*) of Au-Nm (Fig. 1A, ii). Although a slightly decreased reaction with MAL II was observed (Fig. 1A, ii), it is probably due to the unspecific cleavage of α2–3-linked SAs by Au-Nm [23]. From these results, the following experiments were performed by focusing on the Vc-Nm-treated RBCs.

SDS-PAGE analysis of the RBC ghost obtained from the RBCs with or without Vc-Nm treatment showed no difference in the CBB staining pattern (data not shown). However, lectin blot analysis of the ghosts with MAL II showed that three positive bands of 97, 33, and 31 kDa disappeared after the Vc-Nm treatment (Fig. 1B). A band with a strong intensity at 58.5 kDa was observed in the Vc-Nm-treated RBCs. This band resulted from the use of commercial Vc-Nm in this study (data not shown).

Effect of Vc-Nm-pretreated RBCs on the in vitro growth of B. bovis: The relative growth percentage of *B. bovis* was assessed at 24 hr post-cultivation after mixing the 5%-parasitized RBCs with either PBS- or Vc-Nm-treated RBCs. The culture of *B. bovis* with 200-mU/m*l* Vc-Nm-treated RBCs resulted in a significant decrease in the parasite growth (3.08% in the control culture vs. 0.86% in the Vc-Nm-treated culture), *i.e.*, only 28.0% of the control growth (Fig. 2A). Furthermore, confocal laser scanning microscopy with MAL II staining demonstrated that *B. bovis* selec-

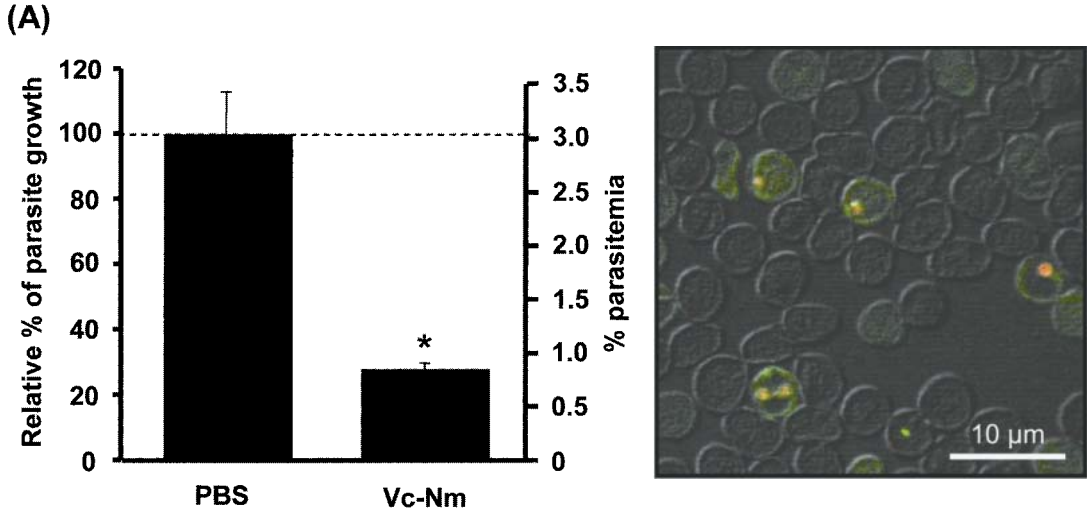

Fig. 2. *In vitro* culture of *B. bovis* in the presence of Vc-Nm-treated RBCs. (A) Relative percentage of *B. bovis* growth assessed at 24 hr after initiating the culture at 1% parasitemia by mixing the 5%-parasitized RBCs with either PBS- or 200 mU/m*l* Vc-Nm-treated RBCs (1:4). Relative values are expressed as the percentage of parasite growth in the culture with Vc-Nm-treated RBCs to that with the PBS-treated control. Error bars stand for the standard deviations (SD). An asterisk is shown above the column with a significant difference from PBS-treated cells ($P<0.05$). An experiment representative of 3 separate experiments is shown. (B) Confocal laser scanning microscopy with MAL II on acetone-methanol-fixed smears obtained from a *B. bovis* culture at 24 hr after initiating the culture with 200 mU/m*l* Vc-Nm-treated RBCs as above. Parasites' nuclei (red) and the reaction of MAL II (green) were visualized with propidium iodide and a combination of the biotinylated MAL II and streptavidin-Alexa fluor 488 conjugate, respectively.

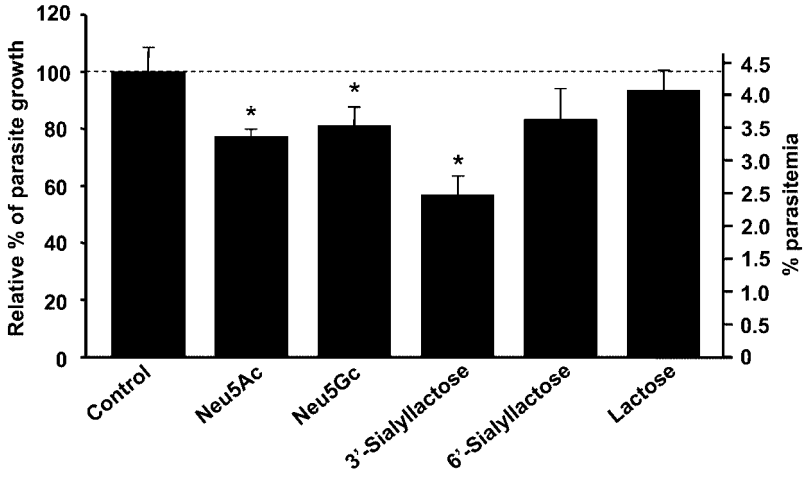

Fig. 3. Inhibitory effects of the exogenous sialic acids and sialyllactoses on the *in vitro* proliferation of *B. bovis*. Parasitemia was determined at 24 hr after initiating the cultures of *B. bovis* at 1.0% parasitemia in the presence of Neu5Ac, Neu5Gc, 3'-sialyllactose, 6'-sialyllactose, and lactose at 1 mM. Data are expressed as the mean relative percentages of parasite growth in a culture with compounds compared to that in a culture without any compounds. Error bars stand for the SD. Asterisks are shown above the columns with a significant difference from the control without any compounds ($P<0.05$). An experiment representative of 3 separate experiments is shown.

tively invaded the intact RBCs harboring the α2–3-linked SA residues on the surface but not the Vc-Nm-treated RBCs (Fig. 2B).

In vitro growth inhibition assay with free SAs and sialyl-conjugates: Neu5Ac is the commonest component of SAs, while Neu5Gc is its 5-position-substituted variant from the acetyl to the hydroxyl base; the latter is not easily detected in healthy humans but is often found in most other mammals [24]. 3'- and 6'-sialyllactoses contain Neu5Ac connected with lactose (Galb(1–4)Glc) via α2–3- and α2–6-linkages, respectively. The control culture showed 4.39% parasitemia at 24 hr. When Neu5Ac or Neu5Gc was added at 1.0 mM, the parasite growth was 3.39 or 3.56%, which indicates significant ($P<0.05$) decrease to 77 and 81% of that without any compounds, respectively (Fig. 3). In addition, 3'-sialyllactose containing α2–3-linked Neu5Ac residues showed a significant 44% reduction of the parasite growth (2.46% parasitemia, $P<0.05$), while 6'-sialyllactose containing α2–6-linked Neu5Ac residues had a slight but not significant inhibitory effect on the growth of *B. bovis*, of which the parasitemia was 3.42% (Fig. 3). Lactose did not show any inhibition (4.06%).

DISCUSSION

The present study provided clear evidence that the α2–3-linked SA residues, but not the α2–6-linked ones, on the surface of bovine RBCs play a crucial role in *B. bovis* invasion. This is concordant with the result obtained by the competitive inhibition assay, which showed a significant inhibition of parasite growth by 3'-sialyllactose but not by 6'-sialyllactose. The unspecific cleavage of the α2–3-linked SAs by Au-Nm shown in an earlier study [23] as well as in the present one (Fig. 1, ii) can explain the contradictory results obtained by Gaffar *et al.* [7], in whose study the single treatments of bovine RBCs with Vc- and Au-Nm showed the subsequent inhibition of parasite growth and a combined treatment showed no additional inhibition. Both enzymes were therefore supposed to cleave the α2–3-linkage of SAs during the pre-treatment of bovine RBCs, which have virtually no α2–6-linked SAs [11].

A variation of the 5-position in SAs is also considered to be important to the specific recognition of host cells by several pathogenic agents [11]. Both Neu5Ac and Neu5Gc inhibited the parasite growth slightly but significantly in the present study (Fig. 3). Although Neu5Gc makes up most of the SAs found on bovine RBCs [18, 19], there is a possibility that only small amounts of Neu5Ac on bovine RBCs are also utilized in the RBC invasion by *B. bovis*. This is supported by the fact that human RBCs, which normally contain Neu5Ac but not Neu5Gc, were more efficiently invaded by *B. bovis* than bovine RBCs [7]. Similarly, it has been reported that EBA 175 of *Plasmodium falciparum*, a human hemoprotozoan parasite showing a pathogenesis similar to babesiosis, requires the Neu5Ac to bind to murine RBCs and participate in the RBC invasion by the parasite [13]. In addition, it is very interesting that 3'-sialyllactose containing α2–3-linkaged Neu5Ac residues showed a considerable reduction in the parasite growth as compared with that of Neu5Ac alone (Fig. 3). These results suggest that *B. bovis* might recognize not only the Neu5Ac *per se* but also the α2–3 linkage to the internal galactose residue as a component of the RBC glycoprotein at the RBC invasion.

The removal of the α2–3-linked SAs from the 97-, 33-,

and 31-kDa bands by the Vc-Nm treatment led to a certain inhibition of the subsequent parasite invasion. It is therefore assumed that the three sialoglycoproteins play significant roles in the RBC invasion. In *P. falciparum*, several sialoglycoproteins contained in the human RBC membrane, such as glycophorins A and C, are known as the host receptors for RBC invasion [4, 8, 17, 22]. In addition, our data presented here suggests that while the α2–3-linked SA-dependent pathway is needed for highly efficient invasion by *B. bovis*, the likelihood of alternative pathways cannot be excluded as the inhibition is only partial in all assays. A detailed characterization of not only these host sialoglycoproteins but also other moieties may offer important information for a better understanding of the hitherto unknown parasite-RBC interaction, in which the merozoite surface antigen (MSA)-1, the MSA-2, the rhoptry-associated protein (RAP)-1, the thrombospondin-related anonymous protein (TRAP), and the apical membrane antigen (AMA)-1 have so far been implicated as protozoan ligands in *B. bovis* [30, 31].

In conclusion, our findings indicated that the α2–3-linked SA-dependent pathway is needed for highly efficient invasion of host RBCs by *B. bovis*, and also suggested that there might also be other potential alternative pathways.

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