Bar-Structure in Bovine Erythrocytes Infected with *Theileria sergenti*

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ABSTRACT. The purpose of this study is to elucidate some morphological characteristics of the bar-structure in bovine erythrocytes infected with *Theileria sergenti*. The bar-structure, the veil or the both were observed in infected erythrocytes. Infected bovine erythrocytes were classified into four types according to the included structures. Infected cells containing bar-structures increased with the progress of parasitemia. In Giemsa-stained blood preparations, bar-structures appeared purplish-red and measured a mean of 1.6 μm in length and up to 0.1 μm in width. Bar-structures were usually straight, sometimes S-shaped, and located in the periphery of infected erythrocytes. In the direct fluorescent antibody test with *T. sergenti*-positive bovine serum both piroplasms and bar-structures exhibited fluorescence. However, in the indirect fluorescent antibody test with monoclonal antibodies against piroplasms only piroplasms showed a highly specific fluorescence. Electron microscopy revealed that bar-structures were vesicular in structure, surrounded by a double membrane connected to the host cell membrane.—**KEY WORDS:** bar-structure, erythrocyte, *Theileria sergenti*.

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Earlier reports have established that erythrocytes infected with piroplasms contain both veil and bar-structure in some *Theileria* species [16]. Uilenberg [16], however, reported consistent absence of the veil in erythrocytes infected with the Japanese *T. sergenti* strain. On the other hand, Tayama [15] and Takao [14] observed veils in erythrocytes infected with *T. sergenti* in Japan, concluding that the veil was a degenerative product of hemoglobin. In the more recent experiments, Minami [12] found both veil and bar-structure in bovine erythrocytes infected with *Theileria* in this country. The change in the course of infection and the morphological features of the bar-structure, however, have not been investigated. The present study, by light and electron microscopies, elucidated some morphological characteristics of the bar-structure in bovine erythrocytes infected with *T. sergenti* and shows the development

of bar-structures in experimental infections.

MATERIALS AND METHODS

Experimental procedure: Five Holstein-Friesian calves of 3–4 months old were inoculated intravenously with parasitized erythrocytes ($5 \times 10^7/\text{kg}$, body weight). During the first 8 weeks following inoculation, each calf was examined once a week for clinical signs, hematological findings, parasitemia and development of veil and bar-structure in the erythrocytes.

The strain of *T. sergenti*: The Chitose strain was used in this experiment. It was isolated by the authors from naturally infected cattle in Hokkaido in 1980. It has been maintained in our laboratory by blood passage to splenectomized cattle.

Observation by light microscopy: Blood smears from each calf were stained with Giemsa. By light microscopy, bar-structures

in infected erythrocytes were examined for color, shape and position in the cell. In addition, blood smears were examined by the direct fluorescent antibody test (FA) with positive bovine serum collected from an experimentally infected calf. In the indirect fluorescent antibody test (IFA) three monoclonal antibodies against piroplasms prepared according to Kobayashi *et al.* [9] were used as anti-serum.

Observation by electron microscopy: Infected blood was fixed in cold 2.5% (v/v) glutaraldehyde, buffered with 0.1 M phosphate at pH 7.4 for 4 hr, and refixed in 1% (w/v) osmic acid for 1 hr. Fixed specimens were dehydrated in ascending alcohol and propylene oxide, and embedded in Quetole 812. The ultrathin sections were stained first with uranyl acetate and secondary with lead citrate, and examined with a JEM-100S transmission electron microscope.

RESULTS

Change in the percentages of bar-structures and veils: In the course of the experimental infections of calves, parasitemia increased through the 5 weeks after inoculation to peak at approximately 9.38% (Fig. 1). Packed cell volume was, approximately 30.2% 1 week, and then gradually decreased to the lowest value of about 14.5%, 6 weeks after inoculation. Bar-structures were formed in approximately 7.0% of *T. sergenti*-infected erythrocytes 1 week after inoculation, and the number of erythrocytes with bar-structures increased with the progress of parasitemia, peaking 4 weeks after inoculation and after that fluctuated irregularly. Veils appeared in approximately 6.8% of the erythrocytes infected with *T. sergenti* 1 week after inoculation and in approximately 5.5–21.9% of infected erythrocytes throughout the experimental period (Fig. 1).

Observation by light microscopy: Infected

erythrocytes were classified into four types: Type I with bar-structures (Fig. 2-A); Type II with veils (Fig. 2-B); Type III with both bar-structure and veil (Fig. 2-C); and Type IV, without any of these structures (Fig. 2-D). No morphological difference was observed between piroplasms in erythrocytes without bar-structures and those in the blood cells with bar-structures and veils.

By Giemsa staining, bar-structures and veils could be differentiated morphologically, because bar-structures were stained purplish-red, whereas veils were dark brown. Bar-structures were usually straight but sometimes S-shaped, measured from 0.8 to 3.4 μm in length with a mean of 1.6 μm , and up to 0.1 μm in width. They were seen in

Fig. 1. Changes in percentages of the 4 types of infected erythrocytes, packed cell volume (PCV), and parasitemia in inoculated calves.

- : parasitemia
- : PCV
- : Type I with bar-structure
- △—△: Type II with veil
- ▲—▲: Type III with the both
- : Type IV without bar-structure nor veil

Fig. 2. Light micrographs of *T. sergenti*-infected erythrocytes. $\times 1,700$.

P: piroplasm
BS: bar-structure
VE: veil

infected erythrocytes at various sites, however, most frequently in the periphery of the cells. No connection was observed between bar-structures and veils, though sometimes attached to the host cell membrane (Fig. 2-A, C).

The FA test with *T. sergenti*-infected bovine serum showed specific fluorescence in both piroplasm and bar-structure (Fig. 3-A). However, the IFA test with three monoclonal antibodies showed highly specific fluorescence only in piroplasms but not in bar-structures (Fig. 3-B). On the contrary, veils reacted neither to infected bovine serum nor to the monoclonal antibodies respond to piroplasms.

Fig. 3. A. *T. sergenti*-infected erythrocytes stained in the FA test using infected bovine serum. Both piroplasms and bar-structures are stained positive.

B. *T. sergenti*-infected erythrocytes stained in the IFA test using monoclonal antibody to piroplasms. Only piroplasms are stained positive.

Fig. 4. Electron micrographs of *T. sergenti*-infected erythrocytes. $\times 16,000$.

Observation by electron microscopy: Bar-structures were slightly curved, cylindrical or vesicular (Figs. 4 and 5), and were generally located in the host cell cytoplasm along the extreme edge, having a common membrane with that of the cell (Figs. 6 and 7). Clearly, the membrane of vesicular bar-structures were a double and the same as of the erythrocytes (Figs. 4 and 7). Other observations also revealed that an erythrocyte-derived membrane enveloped merozoites (parasitophorous vacuole, Fig. 8) and

Fig. 5. Electron micrographs of *T. sergenti*-infected erythrocytes. $\times 42,000$.

that an erythrocyte was invaginated at the membrane by the parasite (Fig. 9).

DISCUSSION

Barnett [4] reported a *Theileria* species of cattle in the eastern USSR, Japan, and the southern and eastern China as *T. sergenti*, and considered its pathogenicity low. Minami *et al.* [11] compared the Japanese and the Russian *Theileria* strains clinicopathologically and serologically, and identified the Japanese strain as *T. sergenti*. However, Uilenberg [16] and Uilenberg *et al.* [17] recommended to apply the name *T. orientalis* to the organism. They reported that in the IFA the four nonpathogenic *Theileria* strains (Japanese and Russian, the Australian, the British, and the U.S.A. strains.) were indistinguishable from each other. Moreover, they reported that piroplasms of these strains were associated with intra-erythrocytic bar-structures, and that veil structures were observed only in infected erythrocytes infected with the British and

Fig. 6. Electron micrographs of *T. sergenti*-infected erythrocytes. $\times 14,000$.

the Australian strains, never in those with the U.S.A. and the Japanese strains [16]. In contrast to these previous reports, however, the present investigation revealed that both bar-structure and veil were present in the

Fig. 7. Electron micrographs of *T. sergenti*-infected erythrocytes. $\times 22,000$.

Fig. 8. Electron micrograph of an erythrocyte-derived membrane containing *T. sergenti* (parasitophorous vacuole). $\times 33,000$.

Fig. 9. Electron micrograph of invagination by *T. sergenti*. $\times 56,000$.

Japanese strain. The present findings may provide key informations for the taxonomy of *T. sergenti*.

According to Young *et al.* [18], bar-structures and veils were seen in eland erythrocytes infected with *T. taurotragi* and piroplasms were connected to bars by a thread-like structure, though "articulated connection" was observed between veils and piroplasms. In contrast, the present study indicated that in *T. sergenti*-infected erythrocytes bar-structures and veils were usually not connected to each other, nor to piroplasms. Bar-structures in infected eland erythrocytes appear a vesicular structure lying in contact with the opening in erythrocytic membrane to the exterior, that is, the bar-structures of the eland *Theileria* are a small vesicle and a part of piroplasms. In the present experiment bar-structures were located in the periphery of host cells, and its membrane continued to that of host cells, as in *T. taurotragi*. However, in contrast to *T. taurotragi*, neither parasite cytoplasm in the vesicular structure nor a connection between bar-structures and piroplasms was observed in *T. sergenti*.

Young *et al.* [18] observed the structures in eland erythrocytes by immunofluorescent complement fixation staining with positive eland serum, and the serum reacted only to piroplasms. Minami [12] reported that in the IFA test with *T. sergenti*-positive bovine serum fluorescence was shown in bar-structures and piroplasms, but in the IFA with immunized rabbit serum against purified piroplasm antigen, fluorescence appeared only in piroplasms. In our experiment, infected bovine serum reacted to both piroplasm and bar-structure, whereas monoclonal antibodies against piroplasm reacted only to piroplasms. These results demonstrate that bar-structures of *T. sergenti* has antigenicity to infected cattle, but not to piroplasms.

The tubule structure was observed in

merozoites of *T. sergenti* [7, 8] and *Babesia equi* [6]. It has been speculated that the function of this cell organ is excretion in *T. sergenti* and ingestion in *B. equi*. But tubule structures morphologically differ from bar-structures of *T. sergenti*.

Bodammer and Bahr [5] postulated that the vesicular structures found in erythrocytes infected with *Plasmodium berghei* NYU-2, were a metabolic window. Aikawa [1] observed cytoplasmic clefts in *P. falciparum*-infected erythrocytes and suggested that they were a route of intracellular transport from the parasites to the erythrocyte membrane. Previous studies demonstrated that invasion by some *Plasmodium* species inflicts changes in the host cell membrane [2, 3, 10], and a similar penetrating process was seen in *Babesia microti* [13]. In the present experiment invagination by the parasite and vesicular bar-structures were observed in erythrocytes infected with *T. sergenti*, this demonstrating that bar-structures in *T. sergenti*-infected erythrocytes may be associated with invasion by parasites. Further studies will be necessary to clarify the formation process and biological function of bar-structures.

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要 約

Theileria sergenti 感染赤血球内における bar-structure の光顕および電顕的観察：川合 覚・高橋清志¹⁾・川本哲¹⁾・永原昌子¹⁾・其田三夫¹⁾・黒沢 隆¹⁾・小沼 操²⁾（食品薬品安全センター泰野研究所，¹⁾酪農学園大学家畜内科学教室，²⁾家畜微生物学教室）——*Theileria sergenti* 感染赤血球を観察した。*T. sergenti* 寄生の末梢赤血球内には bar-structure および veil が認められ，これらの有無により感染赤血球をタイプ I（bar-structure を有するもの），タイプ II（veil を有するもの），タイプ III（bar-structure と veil を有するもの），タイプ IV（両者とも認められないもの），に分けることができた。タイプ I およびタイプ III の感染赤血球は，寄生率の上昇に伴い増加する傾向が見られた。bar-structure はギムザ染色により明瞭な紫赤色に染色され，直線状のものが多く希に湾曲しているものも観察された。bar-structure は赤血球の辺縁に位置することが多く，虫体，veil はそれぞれ分離していた。*T. sergenti* 陽性牛の血清を用いた蛍光抗体間接法による観察で bar-structure は，強い特異蛍光を示したが，1 次抗体として虫体に対するモノクローナル抗体を用いた場合，bar-structure に特異蛍光は認められなかった。電顕的観察において，bar-structure の周囲は赤血球膜と連続した膜構造を有し，その内部構造は空胞状であった。