Abnormality of Osmotic Fragility and Morphological Disorder of Bovine Erythrocytes infected with *Theileria sergenti*

Yukio YAGI, Susumu FURUUCHI¹⁾, Hideyuki TAKAHASHI, and Hiroyuki KOYAMA²⁾

Tohoku Branch Laboratory, National Institute of Animal Health, Shichinohe, Kamikita, Aomori 039-25,
¹⁾Biological Products Division, National Institute of Animal Health, Tsukuba, Ibaraki 305, and ²⁾Department of
Infectious Diseases, School of Veterinary Medicine and Animal Science, Kitasato University, Towada, Aomori 034,
Japan

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ABSTRACT. The osmotic fragility and the surface structure of erythrocytes obtained from 3 calves infected with *Theileria sergenti* and from 3 phlebotomized ones were compared. As the parasitemia progressed, the osmotic fragility of the erythrocytes significantly increased in the infected calves. Particularly the hemolysis ratio in the isotonic area (21.5–94.1%) obviously increased. On the other hand, the percentage of parasitized cells in the erythrocytes did not show so much high values (16.1–21.3%). Similar phenomenon was found in each different percentage of erythrocytes suspension which was separated from density gradient centrifugation. No significant difference in the serum osmotic pressure between the infected calves and the phlebotomized calves was found. By scanning microscopy, the erythrocytes of infected calves, which were collected at the crisis period of parasitemia, were almost completely deformed and showed echinocyte form. Moreover, the appearance ratio of echinocyte form in the erythrocytes population was superior to the percentage of parasitized erythrocytes. Similar membranous alterations were also observed in the erythrocytes of grazing cattle in the crisis period of the theileriosis. It was proven that abnormality of osmotic fragility and morphological disorders of erythrocytes occurred not only in parasitized erythrocytes but also in non-parasitized ones in *T. sergenti* parasitemia.—**KEY WORDS:** echinocyte, erythrocyte, osmotic fragility, *T. sergenti*.

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Bovine theileriosis is one of the most serious diseases of grazing cattle in Japanese pastures, and produces enormous economical damage due to the anemia resulting from it. Though the mechanism of the anemia has not yet been fully elucidated, it has been suggested that it might be due to erythrophagocytosis from immunemediated mechanisms [3]. The anemia of theileriosis seems to resemble that found in malaria infections, because the parasitemia is increased by splenectomy and hemolysis of peripheral blood is rarely detected in infected cattle.

In malaria infection, it has been observed that the membrane structure of erythrocytes is altered by intraerythrocytic parasitism [7], and the susceptibility of parasitized erythro-

cyte membrane to low osmolar condition is higher than in normal ones [6, 9]. Some investigators have suggested that rheological alteration of the erythrocyte membrane is requisite for the development of anemia [5, 13]. Furthermore, Gupta *et al.* [1] reported that these membrane alterations occurred not only in parasitized erythrocytes but also in non-parasitized cells, which they suggested as a possible cause of the anemia. However, very little is known about the osmotic properties and surface structure of *T. sergenti*-parasitized and non-parasitized erythrocytes. Therefore, in this paper we investigated this subject using the osmotic fragility test and scanning electron microscopy (SEM) and presented some interesting results of the alteration of the

erythrocyte membrane in *T. sergenti* parasitemia.

MATERIALS AND METHODS

Experimental animals: Six Holstein splenectomized calves were divided into 2 groups infected and phlebotomized. The infected group consisted of 3 calves (Nos. 2, 6 and 8) which were infected subcutaneously with *T. sergenti* (Fukushima strain: 12.8×10^8 – 21.2×10^8 parasitized erythrocytes). After infection, the calves were treated daily with commercial dexamethazone (DEXAMETHASONE INJ. Nippon Zenyaku Co., Koriyama, Japan) to enhance the parasitemia. The doses of dexamethazone were 0.15–0.30 mg/kg body weight per day and the dexamethazone treatment was continued for 3–5 weeks. The phlebotomized group consisted of 3 calves (Nos. 7, 12 and 21) which were treated by the method described in the previous report [11]; *i.e.*, they received the combined treatment of dexamethazone and phlebotomy to make their packed cell volumes (PCVs) drop to the degree of anemia which occurred during the course of parasitemia in the infected group. The doses used in dexamethazone treatment were the same as those in the infected group, and the phlebotomy was done by removing 700–1500 ml of blood from the jugular vein daily. These treatments were continued for 4–5 weeks.

Routine blood examination: Erythrocyte count, PCVs, hemoglobin concentration and counts of parasitized erythrocytes detected in Giemsa-stained thin smears were examined routinely in blood samples. These blood samples were taken at 1–3 day intervals after the infection or at the time of initiation of phlebotomy until the cessation of the experiment.

Determination of the serum osmotic pressure of *T. sergenti*-infected and phlebotomized calves: Osmotic pressure of sera,

which were collected at the time of routine blood examination of *T. sergenti*-infected and phlebotomized calves, was determined by an osmometer (OSM-1, Shimazu Corp. Kyoto, Japan).

Blood samples: Ten ml of heparinized blood were collected from the jugular vein of infected and phlebotomized calves at 2–12 day intervals. Nine ml were used for the assay of the osmotic fragility test and the remainder was used for the samples observed by SEM. Furthermore, *T. sergenti*-infected blood, which was obtained from a calf with dexamethazone-enhanced parasitemia, was applied to the Percoll-Conray density gradient centrifugation [10], and the fractionated erythrocytes obtained were applied to the osmotic fragility test.

Osmotic fragility test: The osmotic fragility test was done by the method of Parpart *et al.* [4] with a slight modification; *i.e.*, 9 ml of the blood were centrifuged at 1,000 rpm for 20 min at 4°C. After removing the plasma and the buffy coat, the pellet was suspended, washed and centrifuged 3 times in 5 ml of ice-cold isotonic phosphate buffered saline (PBS), pH 7.4, 280 mosM/l. After the final centrifugation, the pellet was resuspended in 5 ml of cold-PBS. Then, either 0.05 ml of the washed erythrocyte suspension or 0.05 ml of the density fractionated red cell suspension was added to two groups of glass culture tubes (14×100 mm) containing 5 ml of serial dilution of PBS (0.10, 0.20, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.75 and 0.85%). One group was allowed to settle at room temperature for 30 min (Parpart 30 min) and the other was incubated at 37°C for 24 hr (Parpart 24hr). Each of the treated erythrocyte suspension was then resuspended and centrifuged at 1,000 rpm for 20 min. The absorbancy of the supernatant at 540 nm was determined using a spectrophotometer (Model 228, Hitachi, Ltd., Tokyo, Japan). Comparative absorbancy values were

Fig. 1. Changes in osmotic fragility of erythrocytes by Parpart 24 hr method accompanying *Theileria sergenti* infection.

a: Day after initiation of dexamethazone treatment. b: Percentage of parasitized cells.

obtained by designating the absorbancy in the supernatant of 0.1% PBS as 100% and expressing the other values obtained as a percentage fraction of this.

Preparation of erythrocytes for morphological observation: One ml of blood was added to 50 ml of PBS and centrifuged at 1,000 rpm for 10 min at 4°C. After the centrifugation the pellet was suspended in 1 ml of PBS and fixed in an equal volume of 2% glutaraldehyde in PBS at 4°C for 2 hr. Fixed erythrocytes were then washed with PBS, dehydrated in a graded series of ethanol, critical dried, coated with palladium gold and examined under a scanning electron microscope (Model S-450, Hitachi, Ltd.). Erythrocytes obtained from 3 grazing

cattle at the crisis period of theileriosis were prepared in the same way.

RESULTS

Changes in the osmotic fragility of erythrocytes accompanying *T. sergenti* parasitemia and phlebotomy: Significant differences in the osmotic fragility of erythrocytes were observed between the infected and phlebotomized groups when the Parpart 24 hr method was employed, but no such clear differences were observed when the Parpart 30 min method was employed. Therefore, only the data from the Parpart 24 hr method were employed in the following. The osmotic fragility changes occurring in the infected group are shown in Fig. 1. As the parasitemia progressed, a significant increment in the osmotic fragility of erythrocytes was observed during the crisis period. Particularly, the hemolysis ratio in the isotonic area obviously increased. That is, the hemolysis ratios of 0.85% PBS (isotonic area) in B-2, 6 and 8 were 91.3% (at 30 day), 94.1% (at 34 day) and 21.5% (at 25 day), respectively. Percentages of parasitized erythrocytes in these calves at each day showed 16.1%, 21.3% and 18.7%, respectively. This phenomenon was not inhibited by the addition of either 22.2 mM glucose in PBS or uninfected serum; moreover, it was not induced by the addition of infected serum to normal erythrocytes (unpublished data).

Changes in the osmotic fragility of the erythrocytes in the phlebotomized calves are shown in Fig. 2. In contrast to the result in infected calves, the osmotic fragility of the erythrocytes in all 3 phlebotomized calves decreased according to the phlebotomizing treatment.

Osmotic fragility of *T. sergenti*-infected erythrocytes separated from the density fractionation: The finding that the hemolysis ratio of 0.85% PBS in infected erythrocytes was superior to the percentage of parasi-

Fig. 2. Changes in osmotic fragility of erythrocytes by Parpart 24 hr method accompanying phlebotomy. Data show the results from one (No. B-7) of the 3 phlebotomized calves. Patterns of the other 2 calves are similar to this one. a: Day after initiation of phlebotomized treatment.

Fig. 4. Scanning electron micrograph of the peripheral blood of a calf (No. B-6) experimentally infected with *Theileria sergenti* (parasitemia; 24.4% PCV; 17.0%). Almost all erythrocytes changed to the echinocyte-like form. $\times 1500$.

tized cells indicates that the increment of osmotic fragility occurred not only in the *T. sergenti*-parasitized erythrocytes but also in the non-parasitized ones. In order to confirm this point, a similar test was performed on each different percentage parasitized erythrocyte suspensions obtained by the Percoll-Conray density gradient centrifuga-

Fig. 3. Osmotic fragility of the *Theileria sergenti*-infected erythrocytes recovered from density gradient fractionation. Data show the results of calf No. B-6 (parasitemia; 14.6%). a: Percentage of parasitized cells.

tion (Fig. 3). Though the percentage of parasitized cells in each suspension ranged from 6.2 and 26.2%, the hemolysis ratio of each suspension in 0.85% PBS showed a high level (above 40%).

Morphological observation of erythrocytes according to the T. sergenti parasitemia: SEM observations of erythrocytes obtained from an infected calf were shown in Fig. 4. At the onset of infection, the erythrocytes showed a normal morphological structure, the discocyte form. As the parasitemia progressed, however, almost all of these erythrocytes changed to the echinocyte form. Erythrocytes obtained from the 3 grazing cattle at the crisis period were altered mostly to the echinocyte form (Fig. 5). In the phlebotomized group, the erythrocytes revealed the discocyte form and did not change (Fig. 6). The osmotic pressure of sera in the infected and the phlebotomized group did not differ between the two groups.

DISCUSSION

Examination of calf erythrocytes infected with *T. sergenti* from the view point of hemolytic behavior and morphological abnormality provided more information concerning the cause of anemia observed in

Fig. 5. Scanning electron micrograph of the peripheral blood of grazing cattle infected with *Theileria sergenti* (parasitemia; 2.6% PCV; 13.0%). Almost all erythrocytes changed to the echinocyte-like form. $\times 1500$.

bovine theileriosis. In this paper, we found that osmotic fragility and deformity of erythrocytes of infected calves increased during the developing course of parasitemia. In *Plasmodium* infection, Sherman [7] reviewed that the morphological and molecular structure of parasitized erythrocytes was altered due to the intraerythrocytic parasitism of malaria parasites. Furthermore, it was reported that erythrocytes parasitized with malaria are less resistant to hypo-osmotic stress than normal erythrocytes [6, 9]. In *Theileria sergenti*-infection, Higuchi *et al.* [2] observed that the surface structure of erythrocytes was morphologically altered to resemble acanthocyte, keratocyte and kinizocyte, concomitant with the developing course of parasitemia. They admitted that this phenomenon depended upon the parasitized erythrocytes. The results of the present density gradient fractionation and SEM observations revealed that these membranous alterations of

Fig. 6. Scanning electron micrograph of the peripheral blood of a phlebotomized calf (No. B-7; PCV; 17.0%). Erythrocytes show the normal discocyte form. $\times 1500$.

erythrocytes occurred also in non-parasitized cells. Furthermore, the erythrocyte membrane of the phlebotomized calves was normal, and the serum osmotic pressure of the infected and phlebotomized calves was consistent: These were not dependent on the mobilization of immature cells in response to developing anemia nor the probable fluctuation of osmotic pressure in serum that might occur during the progress of parasitemia. Since similar morphological alterations in infected erythrocytes were also observed by phase contrast microscopy (unpublished data), it is hardly considered that these alterations were artificially produced under the influence of the preparation of SEM. The doubt, however, remains whether the alterations actually occurred *in vivo*, because above mentioned findings were no more than discoveries observed *in vitro*, so that other factors including suspended medium (PBS), could not be ignored. Still, it is certain that erythrocytes fall into an easily deformed condition with

the development of theileriosis.

The question whether the non-parasitized erythrocytes have sustained damage like the parasitized erythrocytes is significantly important to resolve the mechanism inducing the anemia. In *Plasmodium knowlesi* infection, Gupta *et al.* [1] observed both an alteration of membranous phospholipid component and a defect in the cytoskeletal network in non-parasitized erythrocytes, and they suggested that these phenomena caused the animal to be more anemic. Wright [12] examined the osmotic fragility of bovine erythrocytes infected with *Babesia argentina* and *B. bigemina* and reported that non-parasitized cells were markedly fragile in *B. argentina* infection and that spherocytes were indicated in all the infected erythrocytes. Furthermore, in *B. bigemina* infection, both parasitized and non-parasitized cells were more fragile than normal cells.

Echinocyte form results from the externalization of erythrocyte membrane, which is caused by some factors such as alteration of intraerythrocytic level of ATP or Ca^{2+} and abnormality of membrane cytoskeleton or lipid bilayer. These erythrocytes gradually lose the part of the spine, reduce surface area and finally fall to spherocyte form [8]. Furthermore, it was observed that erythrocytes falling into the above morphological condition were eliminated rapidly from circulation [8].

The results obtained in this study suggest that the same phenomena observed in *P. knowlesi* and babesiosis might occur in *T. sergenti* infection, and that the anemia of this disease might be due not only to the specific trapping of parasitized erythrocytes but also to the extensive clearance involved in non-parasitized cells.

However, as described above, it is hard to determine the morphological structure of

erythrocytes *in vivo*. In order to clarify this question, further studies, including an assay of membrane components such as phospholipids or proteins, should be conducted.

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要 約

Theileria sergenti 感染にともなう牛赤血球の浸透圧脆弱性および膜形態の変化：八木行雄・古内 進¹⁾・高橋秀之・小山弘之²⁾（農林水産省家畜衛生試験場東北支場，¹⁾ 製剤研究部，²⁾ 北里大学獣医伝染病学講座）——*Theileria sergenti* 感染牛赤血球の浸透圧脆弱性および膜形態の変化について検討した。実験感染牛では，原虫寄生率の増加にともない赤血球浸透圧脆弱性は亢進し，等張域における溶血率は原虫寄生率よりも明かに高い値を示した。この現象は密度勾配分画された各寄生率の赤血球においても同様であった。血清浸透圧は感染牛，瀉血牛ともほぼ一定の値で推移した。走査型電子顕微鏡による観察の結果，重篤時の牛の赤血球は変形し，ほとんどの赤血球は Echinocyte の状態に陥っていることが判明した。また Echinocyte の比率は原虫寄生率よりも著しく高い値を示した。同様の現象は小型ピロプラズマ病重篤時の放牧牛においても認められた。以上の成績から，赤血球の浸透圧脆弱性と膜形態の異常は *T. sergenti* 寄生赤血球のみならず，非寄生赤血球においても起きていることが示唆された。