NOTE Clinical Pathology

Increase in Oxidized Proteins in Theileria sergenti-Infected Erythrocyte Membrane

Yukio YAGI, Prasobporn THONGNOON, Hiroki SHIONO and Yukio CHIKAYAMA

1Hokkaido Research Station, National Institute of Animal Health, Sapporo, Hokkaido 062-0045, Japan and 2Southern Veterinary Research and Diagnosis Center, Thung Song 80110, Thailand

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ABSTRACT. As a part of the elucidation of the pathogenesis of anemia in Theileria sergenti infection, oxidized-erythrocyte membrane proteins (OEMPs) collected from T. sergenti-infected calves were examined. The amount of OEMPs were seen to increase with the progress of the anemia and showed a maximum value around the crisis period of the infection. The increase of OEMPs coincided with band Nos. 1, 2, 2.1, 3, 4.1, 5, 6, and 7. The majority of them was located at the Triton X-100 un-extractive phase, and was confirmed as cytoskeletal proteins. This evidence indicates the enhancement of erythrocytic oxidation, and suggests that it might be one of the aggravating factors of anemia in T. sergenti infection.

KEY WORDS: anemia, oxidized protein, Theileria sergenti.

Bovine theileriosis, which is caused by T. sergenti infection, is one of the most serious diseases in grazing cattle in Japan. The main symptom of this disease is anemia; however, the pathogenesis of this anemia is not clear. It has been reported that abnormal osmotic fragility and morphological erythrocyte disorder were observed according to parasitemia [17], that the erythrocyte survival rate declines with the parasitemia [18], and that these phenomena occur in the both parasitized and un-parasitized erythrocytes [18].

The erythrocyte has several intracellular modes of protection against oxidation. For example, it carries enzymes such as superoxide dismutase, glutathione peroxidase, and catalase capable of disarming oxidizing radicals, and it contains antioxidants such as reduced glutathione (GSH) and vitamin E [4]. However, in certain hereditary disorders, excessive oxidant generation, or altered cellular metabolism, decreases levels of GSH or vitamin E and can lead to oxidant damage to membrane lipids and proteins. Such altered physiology and subsequent oxidant damage has been reported in sickle cell disease [6, 10, 13] and glucose-6-phosphate dehydrogenase (G-6-PD) deficiency [9]. It has been reported that oxidative injury to erythrocytes increases their removal by macrophages [3].

Oxygen-derived free radicals have been implicated to serve important roles in severe infection including protozoan infections [5, 8, 16]. In a previous report, the level of plasma vitamin E showed a minimum value when PCV decreased to its minimum value in anemic cattle infected with T. sergenti [19]. More recently, methemoglobin (MetHB), which is an oxidized form of hemoglobin, increased according to the onset of anemia [14], and also decreased in erythrocyte GSH and G-6-PD after T. sergenti infection (unpublished data). In the present study, the oxidative condition of erythrocyte membrane proteins in T. sergenti infection was investigated in order to elucidate the pathogenesis of this anemia.

Three Holstein splenectomized calves (Nos. 851,869, and 870) were inoculated subcutaneously with gland homogenates of T. sergenti-infected ticks (Haemaphysalis longicornis). Ten ml of heparinized blood samples were collected from the jugular vein at 2–3 day intervals and centrifuged at 3,000 rpm for 15 min at 4°C. After the plasma and buffy coat were removed, the pellets were suspended in ice-cold phosphate-buffered saline (PBS), pH 7.4, and washed 3 times in ice-cold PBS. Contaminated leukocytes in washed erythrocytes were counted by an automatic cell counter (NIHON KOHDEN, Celltic MEK-5153, Tokyo, Japan). T. sergenti-infected erythrocytes were lysed by 20 millimolars/l phosphate buffer, according to the method of Dogde and Phillips [7] and hemoglobin-free erythrocyte membranes were recovered. Carbonyl groups of erythrocyte membrane proteins, which are taken as presumptive evidence of oxidative modification, were measured using a commercial kit (ONCOR, OxyBlot #S7150-KIT, Michigan, U.S.A.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed by standard methods and HRP probes were detected using a chemiluminescent detection system (Amersham, RPN2106, Buckinghamshire, UK). Samples used in SDS-PAGE were also applied for HPLC (Hitachi Ltd, L-7100, Tokyo, Japan) in order to estimate the carbonyl content in the erythrocyte membranes according to the method of Levine et al. [12]. The chromatograms at 276 nm (protein) and 370 nm (hydrazone) were monitored using a diode-array detector (Hitachi Ltd., L-7450, Tokyo Japan), and the carbonyl content of the erythrocyte-membrane proteins was expressed as moles carbonyl per mole protein (mc/mp). Packed cell volume (PCV) and percentage of T. sergenti-parasitized erythrocytes (parasitemia) in Giemsa-stained blood smears were examined routinely.

Figure 1 shows the changes in oxidized-erythrocyte membrane proteins (OEMPs) of T. sergenti-infected calves. Before infection, the amounts of OEMPs in all 3 calves were detected below 0.2 mc/mp. These values increased with the intensity of the parasitemia and anemia. Figure 2 shows Western blotting profiles of OEMPs in erythrocyte membr-

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branes, which were collected before infection and the crisis period of the infection. Before infection, several DNP reactive bands were observed, which coincided to band Nos. 1, 2, 2.1, 3, 4.1, 5, and 6. At the crisis period of the infection, the intensity of these bands increased significantly, and other bands including band 7, appeared. Triton X-100 extraction was performed in order to characterize these OEMPs (Fig. 3). The majority of OEMPs was detected in the TX-100 un-extractive phase.

In the present study, OEMPs were increased by intensity of parasitemia and anemia and the molecular weights of these OEMPs coincided with bands 1, 2, 2.1, 3, 4.1, 6, and 7. Concurrently, it was confirmed in membrane cytoskeletal proteins by using the Triton X-100 extraction method [15]. The contamination of leukocyte membranes is quite limited, i.e., lower than 0.1% of leukocytes throughout the examination was contaminated during erythrocyte preparations, which is negligible in light of the results regarding the OEMPs. Therefore, the increase of OEMP in *T. sergenti* infection may be derived mainly from the oxidation of membrane cytoskeletal proteins. It has been reported that the production of intracellular oxygen radicals in sickle RBCs is larger than that in normal RBCs and these radicals introduce the oxidation of the RBC membrane; briefly, this oxidation appeared strongly in membrane cytoskeletal protein band Nos. 1, 2, 2.1, and 4.1 [13], and in band Nos. 1, 2, 3, 4.1, 4.2, 5, and 6 oxidized during lipid oxidation [2]. The erythrocyte membrane skeleton has previously been identified with inner surface fibrillar material, and stabilizes the cell shape and maintains the membrane structure [11]. The oxidation of inner surface proteins (skeleton) occurs in *T. sergenti* infection suggests the increase in oxidizing radical productions, and/or the deficient of antioxidant system occur in erythrocytes. In a previous study, it was shown that an increased MetHB concentration in *T. sergenti* infection coincided with anemia [14]. Although the reason for the enhancement of erythrocyte oxidation was not clear, the oxidation of membrane cytoskeleton may causes both structural and functional defects [1], and oxidized erythrocytes may be destroyed easily by erythropagocytosis [3]. It seems likely that the pathogenesis of the anemia associated with *T. sergenti* infection is partially derived from this oxidation of the membrane cytoskeleton. Further studies are required to clarify the reason why this enhancement of erythrocyte oxidation occurs.

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Fig. 3. Oxidized proteins of erythrocyte membrane after Triton X-100 extraction. Lane 1 shows erythrocyte-membrane proteins from T. sergenti-infected calf (No. 851, 33 days after infection, PCV: 15.7%, parasitemia: 20.3%) stained with Coomassie blue. Lanes 2–4 reveal oxidized protein profiles by Western blotting. Lane 2 shows the sample of lane 1, Lane 3 shows the Triton X-100 extractive phase of the lane 2 sample. Lane 4 shows the Triton X-100 un-extractive phase of the lane 2 sample.