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The Influence of Oxidative Bursts of Phagocytes on Red Blood Cell Oxidation in Anemic Cattle Infected with *Theileria sergenti*

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The primary clinical symptom of Japanese bovine theileriosis, caused by the intraerythrocytic protozoan *Theileria sergenti*, is anemia, but the underlying mechanism of this anemia remains unknown. To elucidate the pathogenesis of anemia developing in bovine theileriosis, we investigated the relationship between oxidative bursts of peripheral blood phagocytes (neutrophils and monocytes) and the oxidation of red blood cells (RBC) to the development of anemia in cattle experimentally infected with *T. sergenti*. The levels of methemoglobin (MetHb) and malondialdehyde (MDA), as a parameter of intracellular and membrane oxidative damage in RBC and of production of hydrogen peroxide (H_2O_2) in phagocytes, were low before the onset of anemia; these parameters began to increase remarkably with decreasing packed cell volume and increasing parasitemia during the course of the anemia, which returned to initial levels during convalescence from anemia. A positive correlation between H_2O_2 production of phagocytes and each of the oxidative indices of MetHb and MDA was also noted during the onset of anemia. The levels of antioxidants, namely reduced glutathione and glucose-6-phosphate dehydrogenase, in RBC also decreased during the progression of anemia. These results suggest that oxidative damage of RBC has a close relationship with the onset of anemia in bovine theileriosis, and that oxidative bursts of phagocytes may play a part in the pathogenesis of anemia in infected cattle.

Keywords: Anemia; Red blood cells; Oxidation; Oxidative burst; Phagocyte; Bovine theileriosis

INTRODUCTION

Bovine theileriosis is one of the most problematic infectious diseases of grazing cattle in Japan and other East Asian countries.[1,2] This disease is caused by a tick-borne protozoan parasite (*Theileria sergenti*) that is transmitted by bloodsucking tick vectors on grazing lands. The main clinical symptom of this disease is severe anemia due to *T. sergenti* parasitism of red blood cells (RBC). Serious production losses, such as arrested development, reproductive disturbance, and occasional death in severe cases, occur as a consequence of the persistence of chronic anemia. As yet, there are no effective vaccines or drugs that can protect susceptible cattle against *T. sergenti* infection. It is therefore necessary to concentrate on acquiring the fundamental knowledge about the pathogenesis of this type of anemia.

Various theories have been proposed to explain the onset of anemia, but the underlying mechanism of anemia caused by *T. sergenti* infection has heretofore been unclear. Regarding the pathogenesis of this anemia, morphological changes in RBC,[3,4] an increase in the osmotic fragility of RBC,[4] an abnormal acceleration of RBC clearance,[5,6] the existence of hemolytic activity in bovine serum having high parasitemia,[7] and a cellular immune response[8–10] have been observed in *T. sergenti*-infected cattle. Although the above circumstantial evidences have been documented, a definitive relationship between these respective factors and the development of anemia could not be established.

Interestingly, during the onset of anemia, the clearance of both *T. sergenti*-parasitized and

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non-parasitized bovine RBC from peripheral blood is accelerated in infected cattle,[5] and has been demonstrated to be accelerated likewise in a severe combined immune deficiency mouse model for *T. sergenti*.[6] This clearance results in severe anemia and a low rate of parasitized RBC in *T. sergenti*-infected cattle. This observation suggests that non-parasitized as well as *T. sergenti*-parasitized RBC may be injured by an unknown mechanism, resulting in progressive anemia in this disease.

In recent years interest has increased in the study of oxidative stress caused by reactive oxygen species (ROS) that influence the disease process.[11,12] Of all the hemoparasitic agents, malarial parasites are recognized to be the most closely related to induction of oxidative stress.[13–17] We recently found that methemoglobin (MetHb) levels, as an indicator of RBC oxidation, increased markedly with the onset of anemia, and that this increase correlated well with the decrease in packed cell volume (PCV) in cattle experimentally infected with *T. sergenti*.[18] Additionally, we demonstrated that oxidized-RBC membrane proteins increased with the progression of anemia and reached a maximum value at the peak of anemia in *T. sergenti*-infected cattle.[19] These results strongly suggest that RBC oxidation is closely related to the pathogenesis of anemia caused by *T. sergenti*; however, the exact mechanism of development of the oxidative process in RBC remains a mystery.

Oxygen-derived ROS have been suggested to serve important roles in severe infection including protozoan infections.[16,20] In *T. sergenti* infection, the activation of blood macrophages has been shown to increase with parasitemia,[8,21] but the actual role of this activation and its effect on the development of anemia *in vivo* is currently unknown. We speculated that oxidative stress due to phagocytic cells, especially ROS derived from neutrophils and/or monocytes, might be one of the leading causes of RBC oxidation. No previous work, to our knowledge, has focused on establishing the relationship between oxidative damage of RBC and ROS production of phagocytes (neutrophils/monocytes) in *T. sergenti* infection. If ROS derived from phagocytes contribute towards the *in vivo* oxidation of RBC in infected cattle, the ROS production levels in anemia could be expected to be higher than those before the development of anemia. In the present study, we examined the relationship of ROS produced by phagocytes, oxidative damage of RBC, and anemia. Our results indicated the existence of an association between RBC oxidation and ROS production by phagocytes, which could be related to a possible mechanism of developing anemia in bovine theileriosis.

MATERIALS AND METHODS

Experimental Animals

A total of ten healthy male Holstein-Friesian cattle an average of 10 (4–18) months of age at the start of the experiment were used. These cattle were procured from the National Agricultural Research Center for Hokkaido Region and were housed in individual pens (2.7 m × 3.4 m) on a flat concrete floor. The cattle were cared for according to the Laboratory Animal Control Guidelines at the National Institute of Animal Health throughout the experimental period. These cattle were fed twice daily (morning and evening) with commercial concentrate-forage diet and roughage based on the Japanese Standard Feeding for Beef Cattle (Agriculture, Forestry and Fisheries Research Council Secretariat, 2000), and had free access to water and mineral block. One month prior to the parasite inoculation, the cattle were splenectomized in order to intensify the level of parasitemia, and were examined periodically for hematological and clinical signs up to the start of the experiment.

Parasite Stock and Experimental Infection

Ikeda stock of *T. sergenti* was used in this experiment. The parasite was maintained by serial inoculation of parasitized RBC or tick passages by *Haemaphysalis longicornis* ticks into splenectomized cattle in our laboratory. All the cattle were confirmed to be normal by routine blood tests and to be free from any hemoparasite infection by Giemsa-stained blood smears at the start of the experiment. Cattle were infested with 50–60 *T. sergenti*-infected ticks over a two-week period by allowing the ticks to feed on the skin of the external ear using an ear bag (cattle nos. 1–5)[22] or inoculated in the neck subcutaneously with 8–30 ml of the same stock of *T. sergenti*-parasitized RBC (cattle nos. 6–10).[18] Infectivity of the ticks was confirmed in advance by the presence of sporozoites in tick salivary glands.

Blood Sampling and Routine Hematological Examination

Peripheral blood samples of each cattle were collected by jugular venipuncture into evacuated tubes containing ethylenediamine-tetraacetic acid dipotassium salt for routine blood test and into heparin-treated tubes for other analyses. Blood samples were collected every other day, starting before the parasite inoculation and continuing up to the convalescent stage of anemia. The RBC, white blood cell counts, PCV values, and hemoglobin concentrations were measured routinely using an automatic blood cell counter (Model K-1000, Sysmex

Corp., Kobe, Japan). The rate of *T. sergenti*-parasitized RBC, determined by light microscopy with Giemsa-stained blood smears based on the count of parasitized RBC per 2,000 RBC, was expressed as a percentage.

Biochemical Assays of RBC Oxidation

Methemoglobin

MetHb concentrations in RBC of six cattle (nos. 3–5 and 8–10) were spectrophotometrically determined at 630 nm according to the method described by Evelyn and Malloy.[23] The values were expressed as a percentage of the total hemoglobin concentration.

Membrane Lipid Peroxidation

Lipid peroxidation levels in RBC membrane of six cattle (nos. 3–5 and 8–10) were measured by the thiobarbituric acid (TBA) reactive substances test described by Yagi,[24] with a slight modification. In brief, 1.0 ml of the ghost, prepared according to the method of Burton *et al.*,[25] was mixed with 3.0 ml distilled water. The samples were then boiled with 1.0 ml of TBA reagent for 60 min. After cooling in tap water, 4.0 ml of *n*-butanol was added and the lipid peroxides were extracted by shaking vigorously. Then centrifugation was performed, and *n*-butanol layers were determined fluorometrically (ex 515 nm; em 553 nm). Lipid peroxidation levels were expressed as nanomoles of malondialdehyde (MDA) per gram of hemoglobin (nmol/gHb), using 1,1,3,3-tetraethoxypropane as a standard.

Reduced Glutathione

Reduced glutathione (GSH) levels in RBC of four cattle (nos. 1, 2, 6, and 7) were measured spectrophotometrically at 412 nm at 37°C by 5,5′-dithiobis(2-nitrobenzoic acid)-glutathione reductase recycling method of Kondo and Sawada,[26] and were expressed as millimoles per milliliter of RBC.

Glucose-6-phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) activities in RBC of four cattle (nos. 1, 2, 6, and 7) were measured spectrophotometrically at 340 nm at 37°C by monitoring the production of NADPH as described by Beutler,[27] and were expressed as international units per gram of hemoglobin (IU/gHb).

Determination of Oxidative Bursts of Phagocytes

Isolation of Phagocytes

Whole leukocytes including phagocytes were isolated from heparinized peripheral blood by hypotonic lysis. In brief, blood was lysed with ice-cold hypotonic saline solution (0.2% NaCl), and isotonicity was restored by adding hypertonic solution (1.6% NaCl) after 60 s. After centrifugation of the cell suspension, the leukocytes were washed twice in Ca^{2+}, Mg^{2+}, and phenol-red free Hank's balanced salt solution (HBSS, pH 7.4), then gently resuspended at a concentration of 3.0×10^6 cells/ml in HBSS. The cell viability was more than 95% by the trypan blue exclusion test. To avoid phagocyte activation by contact with glass or bacteria, sterilized plastic tubes and working solution were used throughout.

Oxidative Burst of Phagocytes

The oxidative burst activity of neutrophils/monocytes in six cattle (nos. 3–5 and 8–10) was studied using a 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) fluorescein probe (CellProbe™ DCFH, PMA · Oxidative Burst, #7547078, Beckman Coulter, Inc., Miami, FL, USA). The green fluorescence observable by flow cytometry was in proportion to the degree of oxidative burst, (i.e. the level of production of hydrogen peroxide (H_2O_2)).[28] A total of 50 µl of leukocyte suspension (1.5×10^5 cells) in HBSS was incubated in a water bath at 37°C for 10 min. Then 25 µl of enzyme substrate (DCFH-DA) containing a phorbol 12-myristate 13-acetate (PMA; 100 ng/ml, final concentration) as stimulus was added and the cell suspensions were reacted at 37°C for exactly 5 min. After incubation, the reaction mixtures were placed on crushed ice for 5 min, then 1 ml of ice-cold HBSS was added to them. Control samples of phagocytes without DCFH-DA fluorescein probe were prepared in each assay. Samples were kept on ice and immediately analyzed in a flow cytometer.

Flow Cytometry

Data acquisition and analysis were carried out using an EPICS® XL™ flow cytometer equipped with System II™ software version 3.0 (Beckman Coulter, Inc., Miami, FL, USA). Before daily analysis, a quality-control check of the flow cytometer was performed by verifying the stability of the optical and fluidic systems using Flow-Check™ fluorospheres (Beckman Coulter, Inc.). For consistent results, the light-scatter intensity and fluorescence intensity of the instrument was also standardized using Flow-Set™ fluorospheres (Beckman Coulter, Inc.). At least 5,000 cells were examined in each analysis, and leukocyte populations (neutrophils, monocytes, and lymphocytes) were identified and selected by setting a gate in a two-dimensional dot plot of forward/side light-scatter signals. The green fluorescence of

a neutrophilic/monocytic oxidative burst (H_2O_2 production) was detected by a 525 nm bandpass filter. Several contaminating RBC, aggregated cells, and some debris were excluded from the analysis using the forward-scatter threshold. To estimate the oxidative burst of cells, fluorescence histograms were developed on the gated-cell populations, and the mean fluorescence intensity (MFI) values of gated cells were automatically calculated. An oxidative burst of neutrophils/monocytes is expressed as the total amount of H_2O_2 production in 1 μl of peripheral blood by the following equation:

$$\text{Amount of oxidative burst (FI/}\mu\text{l blood)} = \text{MFI} \times \text{neutrophil or monocyte counts/}\mu\text{l blood}$$

Statistical Analysis

In order to estimate the association between respective RBC oxidative variables (MetHb and MDA) and the phagocyte oxidative burst (H_2O_2 production), Spearman's correlation coefficient by rank test (Statcel software, OMS publishing Inc., Tokorozawa, Japan) linked to Microsoft® Excel 2000 was applied.

RESULTS

Parasitological and Hematological Finding

As shown in Fig. 1, *T. sergenti*-parasitized RBC in the peripheral blood were detected in all ten infected cattle on days 9–50 after infection, and the parasitemia (rate of *T. sergenti*-parasitized RBC) gradually increased with decrease in PCV. Although great differences in incubation period (the time from parasite infection to the onset of anemia) were observed among individuals, all the infected cattle showed severe anemia. The changing patterns of increased parasitemia and decreased PCV values were almost similar between the two infection methods (tick vector vs. parasitized RBC). These results are consistent with those reported previously.[18] No additional parasites (i.e. other than *T. sergenti*) were observed by routine microscopic examination of blood smears throughout the experimental period. At the peak of anemia, the minimal value of PCV in the peripheral blood ranged from 10.2 to 18.9%. The whole blood samples collected before and after the peak of anemia were chocolate brown in color indicating the pathognomonic of RBC oxidation. Decrease of both RBC counts and hemoglobin concentrations were also similar to changes in the PCV values in all the infected cattle (data not shown).

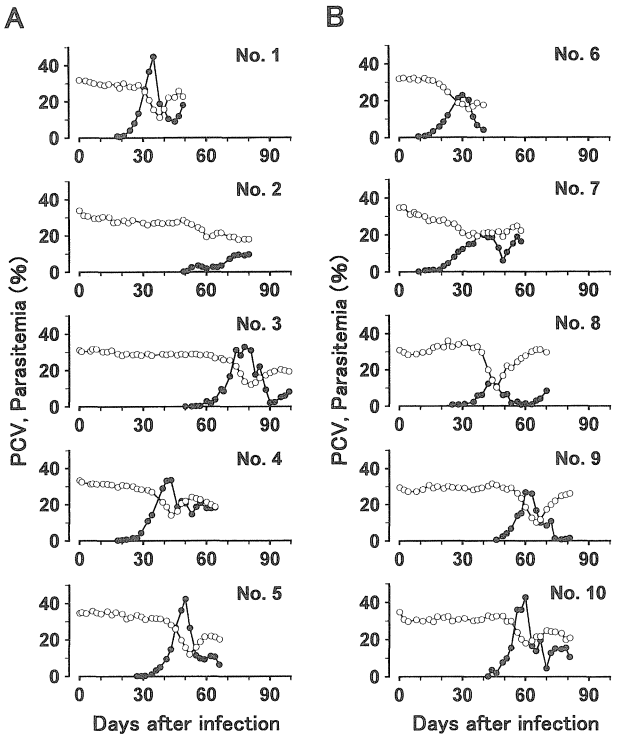

FIGURE 1 Time course of parasitemia (closed circles) and PCV values (open circles) in the peripheral blood of the cattle infected with *T. sergenti*. Five cattle (A) were infested by exposure to *T. sergenti*-infected tick vectors, and the remaining cattle (B) were inoculated with *T. sergenti*-parasitized red blood cells. Parasitemia was evaluated by counting 2,000 red blood cells in Giemsa-stained blood smears. PCV: packed cell volume.

Oxidative Alteration of RBC

Changes in the antioxidative parameters in RBC of four infected cattle (nos. 1, 2, 6, and 7) are shown in Fig. 2. The levels of GSH and activities of G6PD in RBC began to decrease with the progression of anemia after *T. sergenti* infection. Around the peak of anemia, both antioxidants reached their minimal values. The ranges of minimal values of GSH and G6PD around the peak of anemia were 7.5–12.7 mmol/ml RBC and 1.20–2.08 IU/gHb, respectively.

The levels of intracellular and membrane oxidative damage in the RBC of the other six infected cattle (nos. 3–5 and 8–10) are compared in Fig. 3. The contents of MetHb and MDA were low before the onset and during the progression of anemia, though both oxidative parameters were remarkably high around the peak of anemia, and subsequently decreased during the stage of convalescence. The ranges of maximal values of MetHb and MDA at the peak of anemia were 21.6–43.7% and 38.3–348.0 nmol/gHb, respectively.

H_2O_2 Production of Phagocytes

Interesting correlations between the amount of H_2O_2 production derived from neutrophils/monocytes

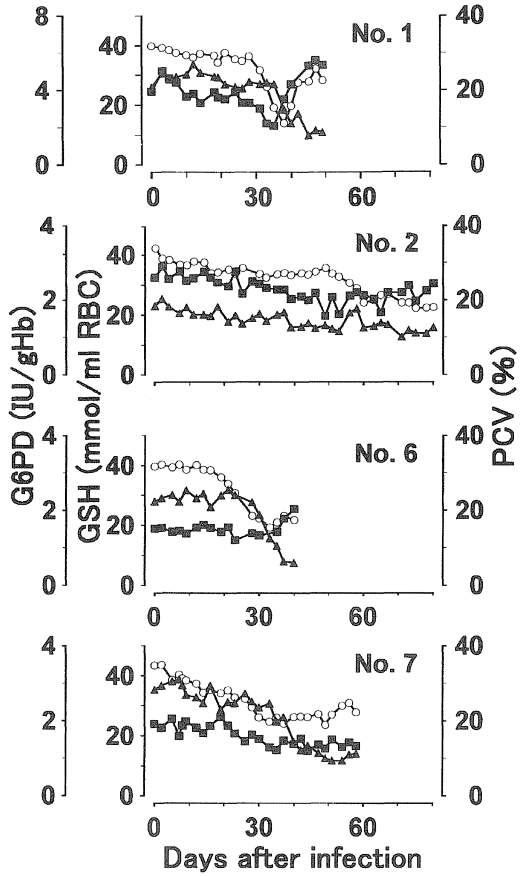

FIGURE 2 Changes in GSH contents (closed triangles), G6PD activities (closed squares) in the red blood cells, and PCV values (open circles) in the peripheral blood of the cattle infected with *T. sergenti*. Two cattle (nos. 1 and 2) were infested with *T. sergenti*-infected tick vectors, and the remaining cattle (nos. 6 and 7) were inoculated with *T. sergenti*-parasitized red blood cells. GSH: reduced glutathione, G6PD: glucose-6-phosphate dehydrogenase, PCV: packed cell volume, RBC: red blood cells, Hb: hemoglobin.

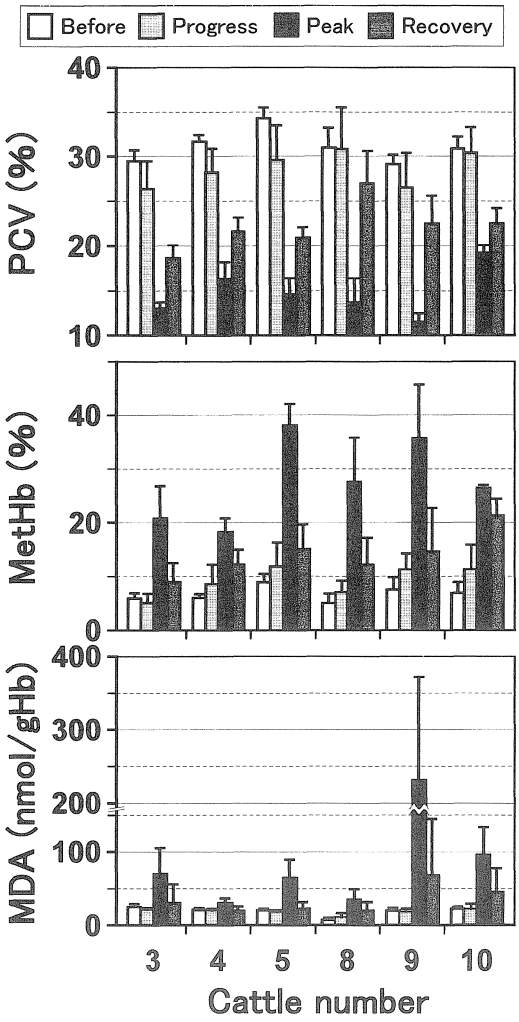

FIGURE 3 Comparison of oxidative damage in the red blood cells and PCV values in the peripheral blood of the cattle infected with *T. sergenti*. Three cattle (nos. 3–5) were infested with *T. sergenti*-infected tick vectors, and the remaining cattle (nos. 8–10) were inoculated with *T. sergenti*-parasitized red blood cells. Values are expressed as mean ± standard deviation of the results from each period, which were divided into the following four stages of the disease: Before represents the stage prior to the appearance of *T. sergenti*-parasitized red blood cells in the peripheral blood and before parasite inoculation in the experimental cattle; Progress represents the progressive stage of the anemia from the appearance of *T. sergenti*-parasitized red blood cells to several days before the peak of the anemia in the infected cattle; Peak represents several days before and several days after the peak of the anemia (4–5 days) in the infected cattle; and Recovery represents the stage of convalescent from the anemia from point of severe anemia in the infected cattle and on. PCV: packed cell volume, MetHb: methemoglobin, MDA: malondialdehyde, Hb: hemoglobin.

and the levels of oxidative parameters in RBC of six infected cattle (nos. 3–5 and 8–10) are shown in Fig. 4. There was a difference in the amount of H_2O_2 production between infection methods before the onset of anemia. Although the amount of H_2O_2 production from neutrophils increased, fluctuating slightly before the onset of anemia, elevation of RBC oxidation was not observed in three cattle infected by tick-vector in this same period (Fig. 4A). In the other three cattle inoculated with parasitized RBC, in contrast, increases in the amount of H_2O_2 production of neutrophils/monocytes was hardly seen before the onset of anemia; consequently, the oxidation of RBC did not take place in the same period (Fig. 4B). During the development of anemia in all infected cattle, the amount of H_2O_2 production from neutrophils/monocytes began to increase markedly in proportion to the increase of MetHb and MDA levels in RBC, then decreased with the decline of both oxidative parameters during convalescence. H_2O_2 production of neutrophils/monocytes and RBC oxidation reached a maximum levels at about the same time in each cattle during severe anemia.

Relationship between H_2O_2 Production and RBC Oxidation

The relationship between the amount of H_2O_2 production and the levels of RBC oxidation in six infected cattle from the time of development of

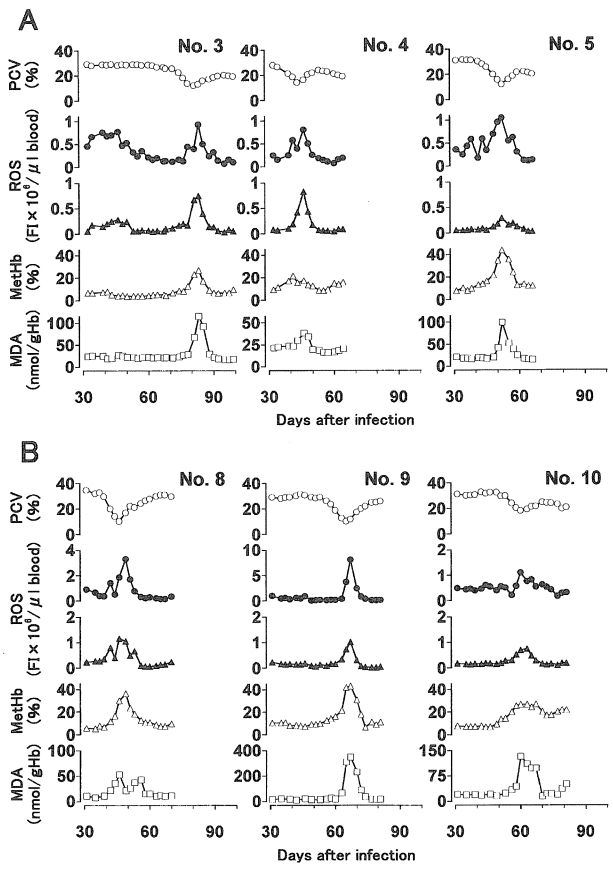

FIGURE 4 Changes in PCV values, the amount of ROS production from neutrophils/monocytes in the peripheral blood, and the levels of oxidative parameters in the red blood cells of the cattle infected with *T. sergenti*. (A) Tick-vector infection (cattle nos. 3–5); (B) parasitized red blood cells used for infection (cattle nos. 8–10). PCV: packed cell volume (open circles), ROS: H_2O_2 production of neutrophils (black circles)/monocytes (black triangles), FI: fluorescence intensity, MetHb: methemoglobin (white triangles), MDA: malondialdehyde (white squares), Hb: hemoglobin.

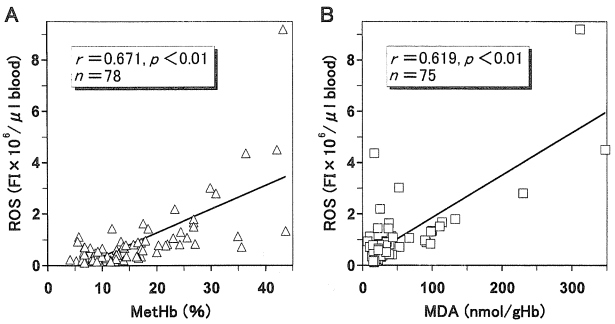

FIGURE 5 Relationship between ROS production from neutrophils/monocytes and oxidative parameters in the red blood cells of the anemic cattle infected with *T. sergenti*. (A) Correlation of MetHb with ROS; (B) correlation of MDA with ROS. Spearman's correlation coefficient by rank test was performed using the values obtained during the development of and convalescence from anemia (26–30 days; average of 28 days) in six infected cattle. MetHb: methemoglobin, MDA: malondialdehyde, Hb: hemoglobin, ROS: H_2O_2, FI: fluorescence intensity.

anemia to that of partial recovery is shown in Fig. 5. The correlation coefficient was evaluated using the sequence of results (12–13 samples per each infected cattle) obtained from the progressive stage to the convalescent stage of anemia. Increase in the amount of H_2O_2 production correlated well with increases in the levels of MetHb ($r = 0.671$; $p < 0.01$) and MDA ($r = 0.619$; $p < 0.01$).

DISCUSSION

The present results indicate that increased H_2O_2 production from neutrophils and monocytes is associated with the degree of RBC oxidation and the progression of anemia in cattle infected with *T. sergenti*. We recently documented that the levels of MetHb, which is an oxidized form of hemoglobin, used as an index of RBC oxidation, markedly increased at the onset of anemia in experimental *T. sergenti* infection, and that the increased MetHb levels correlated significantly with decreased PCV.[18] In addition, we observed increasing oxidized membrane proteins of RBC at the advanced stage of anemia in *T. sergenti*-infected cattle.[19] These results strongly support our hypothesis that RBC oxidation is closely related to the pathogenesis of anemia resulting from *T. sergenti* infection.

Anemia is the most important clinical symptom of bovine theileriosis. During the course of serious anemia in *T. sergenti* infection, rapid and massive clearance of RBC from blood circulation occurs, with little evidence of intravascular hemolysis. Remarkable acceleration of both *T. sergenti*-parasitized and non-parasitized RBC clearance via an unknown mechanism is thought to be the primary cause of the anemia.[5,6] Based on the above circumstantial evidence, we assumed that oxidative injury of RBC may be an important factor in the onset of anemia in bovine theileriosis, and that an oxidative and destructive event may also occur in the RBC during the course of this anemia. To address this issue, we examined the free radical-mediated oxidative damage in RBC of *T. sergenti*-infected cattle.

In the present study, we observed that a decline in PCV is accompanied by dramatic increase in MetHb concentrations and in MDA levels in RBC, indicating that both intracellular and membrane oxidation in RBC occurs in anemic cattle infected with *T. sergenti*. Additionally, oxidative damage of RBC was accompanied by a dramatic and almost simultaneous increase in H_2O_2 production by neutrophils/monocytes, indicating that H_2O_2 production by phagocytes plays a role in the onset of anemia. The obvious question that comes up: as to how a high level of the RBC oxidation is brought about by *T. sergenti* infection. This phenomenon can be explained in three ways.

First, an increase in MetHb levels induces the release of superoxide radicals from hemoglobin[29] and can aggravate oxidative damage to RBC.[29–31] In addition, it is possible that increase in parasite load leads to a decrease in the efficiency of the protective system against oxidative stress in RBC. The malarial parasites, *Plasmodium* spp. have the most intensely examined relationship with free radicals.[14,15,20,32,33] It has been reported that malaria-infected RBC show decreased capacity of their antioxidant enzymes, including superoxide dismutase,[16,34–36] catalase,[16,34–37] glutathione peroxidase,[16,34,36–38] G6PD,[39] MetHb reductase,[34,40,41] and antioxidant substances such as vitamin E,[37,42,43] in RBC. Indeed, in this study, the levels of the antioxidants GSH and G6PD in RBC of infected cattle were observed to decrease parallel with the progression of anemia. Moreover, in the case of cattle infected by the tick-vector method, increase of RBC oxidation was not seen despite the production of ROS (H_2O_2) before the onset of anemia. These results provide circumstantial support for the above-mentioned possibility that an antioxidant mechanism of RBC that protects against oxidative attack may be damaged by *T. sergenti* infection, and that susceptibility of RBC to oxidative stress rises during the development of anemia in infected cattle.

Secondly, the phagocytic cells, namely neutrophils, are capable of destroying a wide spectrum of cell types potentially generative of ROS such as superoxide radical, H_2O_2, hydroxyl radical, and hypochlorous acid.[44–46] Although ROS released from the activated neutrophils play an essential role in host defense against infection, circulating RBC are also extremely oxidation-susceptible, due to the fact that the membranes are rich in polyunsaturated fatty acids and protein composition. Membrane lipids[47] and protein molecules[48] are major targets for cellular damage induced by ROS. In fact, our recent experiment showed that enhanced levels of oxidized proteins in RBC membrane coincided with the onset of anemia in *T. sergenti* experimental infection.[19]

The production of oxygen-derived free radicals[29,32] and reactive nitrogen intermediate[49,50] has been reported in malaria infection. In bovine theileriosis, a few researchers found a slight elevation of H_2O_2 and superoxide radical production,[21] and activation of chemiluminescence response[8] in blood macrophages. However, there are no lines of evidence showing that free radicals play any role in the development of parasitemia and oxidation-related anemia. In an attempt to clarify this causation, we investigated H_2O_2 production in PMA-stimulated neutrophils and monocytes as a possible factor affecting oxidative damage to RBC and the development of anemia following *T. sergenti* infection. In the present study, the number of leukocytes (origin of ROS generation) was observed to increase during severe anemia (data not shown), and a similar result was reported in a previous study also investigating *T. sergenti*-infected cattle.[51]

The findings obtained in our experiment clearly demonstrates that three indices (increased H_2O_2 production of neutrophils/monocytes, enhanced RBC oxidation, and anemia development) occur nearly simultaneously. Moreover, we found a significant correlation between H_2O_2 production from neutrophils/monocytes and an oxidative damage marker in RBC in the anemic cattle, indicating that increased oxidative stress in blood circulation may be an important factor in the onset of anemia in *T. sergenti* infection. Although the precise *in vivo* mechanism of increased H_2O_2 production in neutrophils/monocytes affecting anemia is currently unclear, ROS may directly and/or indirectly play a role not only in the killing of parasites within RBC, but also in destroying both *T. sergenti*-parasitized RBC and non-parasitized RBC in infected cattle. Oxidatively damaged RBC can also promote the phagocytosis of RBC by macrophages.[52,53] It is, thus, likely that RBC are recognized by the macrophages of the reticuloendothelial system and removed from the blood circulation as a consequence of cumulative oxidative injury of RBC induced by *T. sergenti* infection, a process which may be similar to normal physiological clearance such as that of senescent cells. We hope to resolve any remaining uncertainty, and elucidate the actual role of phagocytic cells, in a new study.

Finally, a marked increase in serum iron levels and transferrin saturation have been observed in anemic cattle infected with *T. sergenti* during the development of anemia.[54] In contrast, these parameters increase only slightly in the human[55] and the rat[56] infected with a malarial parasite. Judging from these findings, it can be hypothesized that the physiological iron metabolism may not be functioning normally during the development of anemia in bovine theileriosis. As a result of excess iron availability, the initiation of free radical damage-mediated hydroxyl radical formation[57,58] is possible via the iron-catalyzed Haber-Weiss reaction or the formation of iron-ferryl radicals or other iron-oxygen complexes.[59,60] Therefore, indirect oxidative damage to RBC caused by increased iron and its related substances might occur in the infected cattle at the onset of anemia. Based on these suppositions, we believe that the RBC oxidation and free radical-mediated injury observed in the present study may be important causes of anemia in *T. sergenti* infection.

In summary, the consequences of association between increased levels of H_2O_2 production derived from neutrophils/monocytes and enhanced oxidative damage of RBC on anemia in *T. sergenti*

infection in cattle are herein reported, to our knowledge for the first time. However, to establish a clearer understanding of the mechanism and pathogenesis of anemia in bovine theileriosis, further study is necessary.

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