The Effect of Macrophages on the Erythrocyte Oxidative Damage and the Pathogenesis of Anemia in *Babesia gibsoni*-Infected Dogs with Low Parasitemia

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ABSTRACT. The role of macrophages in the erythrocyte membrane oxidative damage and the pathogenesis of anemia in *Babesia gibsoni*-infected dogs with low parasitemia was investigated. Macrophages derived from peripheral blood monocytes (PBM) from *B. gibsoni*-infected dogs produced significantly higher chemiluminescent responses, indicating the release of reactive oxygen intermediates, than those from non-infected dogs when the cells were subjected to non-specific stimulation with phorbol 12-myristate 13-acetate (PMA) and opsonized zymosan (OZ), or infected dog erythrocyte membranes opsonized with infected dog serum. These results indicate that PBM of *B. gibsoni*-infected dogs with low parasitemia were highly activated compared to those of non-infected dogs. Furthermore, the membrane lipid peroxidation of normal dog erythrocytes incubated with PBM from *B. gibsoni*-infected dogs was significantly higher (p<0.05) than that of erythrocytes incubated with PBM from non-infected dogs when the PBM were stimulated with the opsonized membranes. These results suggest that the oxidative damage of erythrocytes observed in *B. gibsoni*-infected dogs with low parasitemia might be induced, in part, by reactive oxygen species released from the activated PBM. On the other hand, the present study also showed a significant increase (p<0.001) of IgG-bound erythrocytes in *B. gibsoni*-infected dogs compared with such erythrocytes in non-infected dogs. The increase of IgG-bound erythrocytes in infected dogs might reflect the increase of erythrocytes with oxidative damage induced by the infection with *B. gibsoni*. The results of the present study suggest that the increase of IgG-bound erythrocytes in the circulation of infected dogs induce a high degree of erythrocyte loss via immunological phagocytosis by activated macrophages, resulting in severe anemia in spite of low parasitemia.

KEY WORDS: *Babesia gibsoni*, erythrocyte oxidative damage, macrophage.

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Although anemia is the major symptom and cause of mortality in animals with *Babesia* infection, the pathogenesis of the anemia remains unclear. In general, *Babesia* parasites, like *Theileria* and malaria parasites, invade erythrocytes of infected animals, resulting in the destruction of the parasitized erythrocytes. Also, it has been observed that severe anemia often occurs in animals infected with these parasites in spite of a low percentage of parasitized erythrocytes in their peripheral blood [14], which often leads to an erroneous diagnosis. This phenomenon suggests that non-parasitized erythrocytes may also be damaged by an unknown mechanism due to the parasite infection, and that non-parasitized erythrocytes may be removed prematurely from the circulation in infected animals. Indeed, increases of the levels of anti-erythrocyte membrane antibodies and of erythrocyte-bound IgG in dogs infected with *Babesia gibsoni* have been reported [1–3, 9, 11], indicating the immune destruction of both parasitized and non-parasitized erythrocytes. Our previous studies also showed that oxidative damage of erythrocytes was induced by the multiplication of *B. gibsoni* [23], and that non-parasitized erythrocytes were exposed to oxidative stress during infection by *B. gibsoni* [23]. Also, increased erythropagocytic activity of macrophages in *B. gibsoni*-infected dogs was observed [22]. In addition, macrophages ingested not only parasitized erythrocytes but also non-parasitized cells [23]. These previous studies suggested that increased oxidative damage of erythrocytes in *B. gibsoni*-infected dogs may enhance the phagocytosis of the erythrocytes by macrophages through an immunological process, resulting in severe anemia in spite of low parasitemia. However, the mechanism underlying the oxidative process in erythrocytes infected with *B. gibsoni* has not yet been clarified, though one of our studies suggested that the parasites infection might be cause oxidative damage in the host erythrocytes because superoxide anions were produced in *B. gibsoni*-infected erythrocytes in *vitro* [28]. In the present study, we investigated the effect of macrophages on the erythrocyte oxidative damage and demonstrated that membrane lipid peroxidation of erythrocytes incubated with activated macrophages from *B. gibsoni*-infected dogs was significantly higher than that of erythrocytes incubated with macrophages from non-infected dogs, suggesting that the oxidative damage of erythrocytes in infected dogs may be induced by reactive oxygen species released from activated macrophages.

MATERIALS AND METHODS

Experimental animals: In this study, five dogs with chronic infection of *B. gibsoni* were used. These dogs were inoculated with infected blood from *B. gibsoni* carrier dogs. After the inoculation, parasites appeared in the peripheral
blood of all dogs, and parasitemia reached a peak level (2.0–17.2%) at 12–24 days, followed by a decrease of hematocrit values and an increase of reticulocyte counts. The parasitemia decreased to 0.5–1.0% at 30–50 days, and thereafter prolonged low parasitemia (0–0.75%) with slight to moderate anemia was continued for about one year. These dogs were used as dogs with chronic babesiosis. The effect of macrophages on the erythrocyte membrane oxidative damage in these dogs was examined at 12–24 months after the inoculation by infected blood from *B. gibsoni* carrier dogs. Six normal dogs, which were born and raised in our laboratory, were used as controls. All experimental procedures were in accordance with the guideline of the animal use regulation of Hokkaido University.

**Isolation of monocytes from peripheral blood:** Peripheral blood monocytes (PBM) were separated as reported previously [22, 23], and resuspended in RPMI 1640 solution (Bio Whittaker Inc., Walkersville, Maryland, U.S.A.) without phenol red or L-glutamine.

**Preparation of stimulants:** Phorbol 12-myristate 13-acetate (PMA, WAKO Pure Chemical, Osaka) was initially dissolved at 1.0 mg/mL in dimethyl sulfoxide as a stock solution and stored frozen at –30°C until use. PMA solutions were prepared from the stock solution by further dilution in Ca²⁺, Mg²⁺-free Hank’s balanced salt solution (HBSS, pH 7.4) to 2 mg/mL.

Preparation of opsonized erythrocyte membranes: Erythrocyte membranes were prepared as described previously [12]. The resultant membranes were opsonized with serum from infected dogs and non-infected dogs for 30 min at 37°C, and resuspended in HBSS, respectively. The following four kinds of opsonized membranes: normal dog erythrocyte membranes opsonized with normal dog serum (A), *B. gibsoni*-infected dog erythrocyte membranes opsonized with normal dog serum (B), normal dog erythrocyte membranes opsonized with infected dog serum (C) and infected dog erythrocyte membranes opsonized with infected dog serum (D) were used as stimulants.

**Measurement of chemiluminescence (CL) response of PBM:** A *Cyprindina luciferin analog, (2-methyl-6-[4-methoxyphenyl]-3,7-dihydroimidazo[1,2-a]pyrazin-3-one Hydrochloride; MCLA, Tokyo Kasei Kogyo Co., Ltd., Tokyo) was prepared according to Nishida *et al.* [24]. OZ was prepared from the stock solution by further dilution in HBSS to 2 mg/mL.

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**RESULTS**

**MCLA-dependent CL responses of peripheral blood monocytes:** When non-specific stimulants were used, the CL
responses of the PBM from *B. gibsoni*-infected dogs were clearly higher than those of the PBM from normal dogs. Figure 1 shows typical patterns of CL response curves of PBM from normal and *B. gibsoni*-infected dogs after stimulation with non-specific agents OZ and PMA. The CL response of the PBM from a representative infected dog began to increase linearly at 72 sec and 144 sec after the stimulation with OZ or PMA, respectively. The maximal response was obtained at 396 sec in the PBM stimulated with OZ, and at 639 sec in the cells with PMA, followed by a rapid decrease. The parasitemia and hematocrit value of this infected dog was 0.35% and 41%, respectively. Similar results were obtained with the PBM from the remaining infected dogs examined. In these remaining dogs, the hematocrit values and the parasitemia ranged from 0–0.7%, and 18.5–45.0% during the experimental period, respectively.

In contrast, the CL response of the cells from a representative normal dog gradually increased and reached a peak at 366 sec in the cells stimulated with OZ, and at 1260 sec in the cells with PMA, respectively (Fig. 1). The mean CL response of PBM from the remaining infected dogs was significantly (p<0.001) higher than that from normal dogs when the CL response curves of PBM from normal and *B. gibsoni*-infected dogs after stimulation with OZ and PMA. The CL response of the PBM from a representative infected dog began to increase linearly at 72 sec and 144 sec after the stimulation with OZ or PMA, respectively. The maximal response was obtained at 396 sec in the PBM stimulated with OZ, and at 639 sec in the cells with PMA, followed by a rapid decrease. The parasitemia and hematocrit value of this infected dog was 0.35% and 41%, respectively. Similar results were obtained with the PBM from the remaining infected dogs examined. In these remaining dogs, the hematocrit values and the parasitemia ranged from 0–0.7%, and 18.5–45.0% during the experimental period, respectively.

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ations in morphology and in biochemical properties such as increased content of lysosomal enzymes and secretion of toxic intermediates like tumor necrosis factor and reactive oxygen species [5, 10, 16, 20, 29, 30]. The activated cells release greater amounts of superoxide anion for intracellular killing of microorganisms when stimulated [17, 25, 34]. Immunological phagocytosis mediated by IgG receptors is also enhanced in the activated macrophages and the cells become capable of phagocytosis mediated by C3b or IgM antibodies in contrast to non-activated cells [13, 15, 18].

In the present study, blood monocytes from *B. gibsoni*-infected dogs produced a significantly higher CL signal, indicating the release of reactive oxygen intermediates [31], than those from non-infected dogs when stimulated with PMA or OZ (Fig. 2), or with infected dog erythrocyte membranes opsonized with infected dog serum (Fig. 3) *in vitro*. PMA is a soluble stimulant, activates NADPH-oxidase via the activation of protein kinase C in place of diacylglycerol [20]. OZ binds to complement and immunoglobulin receptor (CR3 and Fc receptor) on the cell surface, leading to phagocytosis and activation of NADPH-oxidase [6]. These results indicate that monocytes of infected dogs with low parasitemia were highly activated compared to those of non-infected dogs. Furthermore, membrane lipid peroxidation of normal dog erythrocytes incubated with monocytes from *B. gibsoni*-infected dogs was significantly higher than that of normal dog erythrocytes incubated with monocytes from non-infected dogs when the cells were stimulated with the opsonized erythrocyte membranes (Fig. 4). These results of the present study suggest that the oxidative damage of erythrocytes observed in *B. gibsoni*-infected dogs might be induced, in part, by reactive oxygen species released from the activated monocytes. In malaria, *Plasmodium falciparum*-infected erythrocytes show significantly higher lipid peroxidation following exposure to PMA-activated blood monocytes compared with their monocyte-unexposed counterparts [19]. Thus, it is thought that macrophage secretory products, especially reactive oxygen species, may play a role not only in the inhibition of parasite growth within erythrocytes [4, 7, 8, 30], but also in oxidative damage to parasitized and non-parasitized erythrocytes in dogs infected with *B. gibsoni* as well as malaria parasites.

It has been reported that the host response to *Babesia par-

![Fig. 3. Effects of opsonized erythrocyte membranes on MCLA-dependent chemiluminescence of monocytes from *B. gibsoni*-infected (closed columns) and normal dogs (open columns). Values are expressed as mean ± standard deviation of the mean from 15 experiments. †† p<0.01, compared with the values obtained from normal dogs by means of Student’s t-test. Membrane A: normal dog erythrocyte membranes opsonized with normal dog serum, Membrane B: *B. gibsoni*-infected dog erythrocyte membranes with normal dog serum, Membrane C: normal dog erythrocyte membranes with *B. gibsoni*-infected dog serum, Membrane D: *B. gibsoni*-infected dog erythrocyte membranes with *B. gibsoni*-infected dog serum, cps: counts per second.](image)

![Fig. 4. Lipid peroxidation in erythrocyte membranes incubated with activated (A, B) and non-activated monocytes (a, b) from *B. gibsoni*-infected (A, a) and normal dogs (B, b). The concentration of thiobarbituric acid reactive substances (TBARS) in erythrocyte membranes, an indicator of lipid peroxidation, was measured. Values of activated monocytes (A, B) are expressed as mean ± standard deviation of the mean from five experiments. †† p<0.05, compared with the value obtained from erythrocytes incubated with activated monocytes from normal dogs (B) by means of one-sample t-test. Values of TBARS in erythrocytes incubated with non-activated monocytes from infected (a) and normal dogs (b) were the mean values of two experiments. Column A: erythrocytes incubated with activated monocytes from *B. gibsoni*-infected dogs, a: erythrocytes incubated with non-activated monocytes from *B. gibsoni*-infected dogs, B: erythrocytes incubated with activated monocytes from normal dogs, b: erythrocytes incubated with non-activated monocytes from normal dogs, Hb: hemoglobin.](image)
Macrophages were markedly increased in the liver of study has demonstrated that T lymphocytes and activated cytotes and mature macrophages [30, 35]. A histological asites includes the activation of both peripheral blood mono-ctes and mature macrophages [30, 35]. A histological study has demonstrated that T lymphocytes and activated macrophages were markedly increased in the liver of B. gibsoni-infected splenectomized dogs [35]. Those observations suggest that the oxidative damage of parasitized and non-parasitized erythrocytes caused by the activated macrophages in infected animals may be of a higher than normal magnitude. Indeed, it has been reported that B. gibsoni-infected dogs showed a significant increase of oxidative damage in their erythrocytes compared with that in non-infected dogs [21, 23]. On the other hand, one of our studies also revealed that lipid peroxidation in erythrocytes cultured with B. gibsoni parasites was increased with an increase of parasitized erythrocytes in the culture, and that the superoxide generation in the parasitized culture was significantly higher than that in the non-parasitized culture [28]. These results suggested that oxidative damage was also done to the host erythrocytes by the parasites. From these previous and present studies, we conclude that the oxidative damage of erythrocytes in B. gibsoni-infected dogs is induced not only by the parasites per se, but also by the activated macrophages in infected animals.

The present study also showed a significant increase of IgG-bound erythrocytes in B. gibsoni-infected dogs compared with the level in non-infected dogs (Fig. 5). Similarly, an increase of the erythrocyte-bound IgG level in B. gibsoni-infected dogs has been reported [1–3, 9, 11]. It has been thought that senescent erythrocytes bind antibodies and complement, which in turn promotes the recognition and phagocytosis of such erythrocytes by macrophages [32, 33]. In this respect, it has been demonstrated that an antigenic structure of senescent erythrocytes recognized by antibodies already present in the serum can be generated by the clustering of integral membrane proteins, which induces autolous IgG binding, complement fixation and phagocytosis by human monocytes in vitro [33]. Since hemoglobin denaturation [32], malondialdehyde (an end product of lipid peroxidation) and oxidative cross linking can all lead to formation of integral membrane protein clusters [33], the results of our previous and present studies suggest that the increase of IgG-bound erythrocytes in infected dogs might reflect the increase of erythrocytes with oxidative damage induced by the infection with B. gibsoni. Since activated macrophages show enhanced activity of immunological phagocytosis mediated by IgG receptors [33], the increase of IgG-bound erythrocytes in the circulation of infected dogs is expected to result in a high degree of erythrocyte loss by erythropagocytosis, resulting in severe anemia in spite of low parasitemia. Thus, it is thought that activated macrophages play a central role in the pathogenesis of anemia in dogs infected with B. gibsoni.

Fig. 5. IgG-bound erythrocytes in dogs infected with B. gibsoni. Erythrocytes from B. gibsoni-infected (closed column) and normal (open column) dogs were mixed with rabbit anti-dog-IgG antibody conjugated with fluorescein isothiocyanate and incubated for 30 min. After the incubation, the cells were washed and analyzed using a flow cytometer. Values are expressed as mean ± standard deviation of the mean from five experiments. □ p<0.001, compared with the value obtained from normal dogs by means of Student’s t-test.

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