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Increased Generation of Superoxide in Erythrocytes infected with *Babesia gibsoni*

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ABSTRACT. The present study was conducted to clarify the mechanism underlying the oxidative process in erythrocytes infected with *Babesia gibsoni*. The parasite *B. gibsoni* was cultured together with erythrocytes from normal dogs for 7 days. When parasitemia reached 12.0–13.4% at Day 7, the production of superoxide in erythrocytes was significantly higher in the parasitized culture than in the control culture ($p<0.005$). The concentration of thiobarbituric acid reactive substances (TBARS) in erythrocytes in parasitized culture was also significantly increased compared with the control culture ($p<0.005$), indicating that lipid peroxidation was greater in infected erythrocytes than in non-infected cells. In addition, the rates of superoxide generation in the blood of *B. gibsoni*-infected dogs were also significantly higher than in non-infected dogs ($p<0.001$). These results indicate that superoxide anions are increased in erythrocytes parasitized with *B. gibsoni*, and suggest that oxidative damage, due to lipid peroxidation, might be caused in host erythrocytes by the parasite.
KEY WORDS: *Babesia gibsoni*, oxidative damage, superoxide.

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Babesia spp. are well-known blood parasites that cause severe hemolytic anemia in domestic animals [4]. In general, *Babesia* parasites, as well as *Theileria* and malaria parasites, invade erythrocytes of infected animals, which results in the destruction of the parasitized erythrocytes [3]. However, severe anemia often occurs in animals infected with this parasite in spite of a low percentage of parasitized erythrocytes in their peripheral blood [4, 9]. This phenomenon suggests the possibility of non-parasitized erythrocytes also being damaged due to the parasite infection, resulting in enhanced erythrocyte destruction. We previously reported that concentrations of both methemoglobin and malondialdehyde (MDA), an end product of lipid peroxidation, increase in erythrocytes cultured with *B. gibsoni*, and that *B. gibsoni*-infected dogs with high parasitemia also show significant increases of both methemoglobin and MDA in erythrocytes compared with those in non-infected dogs [10]. These results indicate that oxidative damage to erythrocytes is induced by *B. gibsoni* infection and suggest that active oxygen might be generated by the multiplication of the parasites. Furthermore, erythrocytes cultured with *B. gibsoni* are more susceptible to phagocytosis by bone marrow macrophages from normal dogs than erythrocytes cultured without *B. gibsoni*. In addition, 89 percent of erythrocytes phagocytized by macrophages were non-parasitized erythrocytes [10]. However, it has not been clarified whether the *Babesia* parasite is able to generate reactive oxygen species. The present study was carried out to elucidate the mechanisms underlying the oxidative process in erythrocytes infected with *B. gibsoni*.

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MATERIALS AND METHODS

Three dogs were inoculated with *B. gibsoni*-infected blood from other carrier dogs, and used as *B. gibsoni*-infected dogs in the present study. Five normal dogs were used as controls. During the experimental period, parasitemia of the *B. gibsoni*-infected dogs varied from 0% to 0.45%, and hematocrit value from 15.0% to 40.5%.

B. gibsoni was cultured as reported previously [22] with some modifications. Heparinized venous blood from a normal dog was washed twice with Vega y Martinez (VYM) solution [19], and then washed again twice with RPMI 1640 without phenol red and L-glutamine. After the washing, erythrocytes were resuspended to a final packed cell volume of 5% in a culture medium consisting of 60% RPMI 1640 without phenol red and L-glutamine supplemented with sodium pyruvate (0.1 mg/m*l*), glutamine (0.3 mg/m*l*), sodium bicarbonate (2 mg/m*l*), penicillin (100 units/m*l*), streptomycin (100 μg/m*l*) and 40% heat-inactivated normal dog serum. The erythrocyte suspension was mixed with *B. gibsoni*-infected erythrocytes and incubated at 37°C under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 7 days. Every 24 hr, 60% of the culture supernatant was removed without disturbing the sedimented erythrocytes and replaced with an equal volume of fresh medium. The *B. gibsoni* parasites used in this experiment had been maintained in the culture medium described above at our laboratory.

Thiobarbituric acid reactive substances (TBARS) were quantified by the thiobarbituric acid spectrophotometric assay [12]. On Day 7 after the onset of culture, erythrocytes in the culture medium were collected and were washed twice with physiological saline, then resuspended in the same buffer to yield a hematocrit value of 10%. The concentrations of hemoglobin and TBARS of these erythrocyte suspensions were determined. TBARS were expressed as

nmol/g hemoglobin.

The generation of superoxide in erythrocytes from *B. gibsoni*-infected and non-infected dogs was determined according to Yamato *et al*. [23] with a slight modification. Heparinized venous blood from *B. gibsoni*-infected and non-infected dogs was filtered through a column of α-cellulose/cellulose microcrystalline to remove leukocytes and platelets [23]. The blood was then washed three times with Hank's balanced salt solution without phenol red (HBSS) (pH 7.4) at 4°C and diluted to a concentration of 1.0×10^6 cells/μl by HBSS. Fifty microliters of the erythrocyte suspension was mixed with 450 μl of HBSS containing 50 μM cytochrome *c*. The mixture was incubated for 1 hr at 37°C under low oxygen pressure, 5% CO_2, 5% O_2 and 90% N_2 to prevent autoxidation. After incubation, the mixture was centrifuged at 1,250 × g for 2 min at 4°C. The supernatant was separated immediately and the concentration of superoxide was calculated from the difference of absorbance between 550 nm and 540 nm. The rate of superoxide generation was expressed as the rate of cytochrome *c* reduction (nmol/10^9RBCs/hr). Superoxide generation rates in *in vitro* culture were determined on Day 7 after the onset of culture. The specificity of the reaction was confirmed by complete inhibition of cytochrome *c* reduction following the addition of superoxide dismutase to a final concentration of 10 μM to the incubation mixture.

Erythrocyte superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione S-transferase, and methemoglobin reductase activities were assayed according to Beutler [1], using a spectrophotometer (U-3210, Hitachi Co., Ltd., Japan). Superoxide dismutase and the other enzymes were expressed as U/g hemoglobin and IU/g hemoglobin, respectively.

Reticulocytes were prepared as described by Yamasaki *et al*. [21]. Reticulocyte suspensions were prepared to yield reticulocyte concentrations of 0, 20, 40, and 80%. Measurement of the superoxide production rate of reticulocytes was done as described above.

The significance of the differences was evaluated using Student's *t*-test and the Mann-Whitney test. Correlations between superoxide generation and red cell parameters were evaluated by use of Pearson product moment linear regression analysis.

The sources of chemicals used were as follows. Cellulose microcrystalline was from Merck (Darmstadt, Germany). Cytochrome *c* derived from horse heart, α-cellulose and superoxide dismutase from bovine erythrocytes were all from Sigma Chemical (St. Louis, MO, U.S.A.). RPMI 1640 without phenol red and L-glutamine was from Bio Whittaker Inc. (Walkersville, MD, U.S.A.). Percoll gradient solution was from Amersham Pharmacia Biotech (Uppsala, Sweden). All the other chemicals used were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

RESULTS

Concentrations of TBARS in control and parasitized cultures on Day 7 after the onset of the culture are shown in Fig. 1. During the cultivation, the number of parasitized erythrocytes with *B. gibsoni* significantly ($p<0.01$) increased to 13.4 ± 2.9% (mean ± S.D.) on Day 7, from the initial value (2.3 ± 1.0%) when examined immediately before the onset of the cultivation (Day 0). The TBARS concentration in the parasitized culture (279.9 ± 53.2 nmol/g hemoglobin) was significantly ($p<0.005$) higher than that in the control culture (180.9 ± 40.6 nmol/g hemoglobin).

Superoxide generation in the parasitized culture on Day 7 is shown in Fig. 2. During the cultivation the number of parasitized erythrocytes increased to 12.0 ± 3.6% on Day 7 relative to 2.1 ± 0.9% on Day 0. The superoxide generation in the parasitized culture (262.9 ± 66.0 nmol/10^9RBCs/hr) was significantly ($p<0.005$) higher than that in the control culture (187.4 ± 42.5 nmol/10^9RBCs/hr). In this experiment, reduction of cytochrome *c* was completely inhibited by the addition of 10 μM superoxide dismutase (data not shown).

The superoxide generation in erythrocytes from dogs infected with *B. gibsoni* (479.7 ± 121.4 nmol/10^9RBCs/hr) was significantly ($p<0.001$) higher than in those from non-infected dogs (256.9 ± 42.5 nmol/10^9RBCs/hr) (Fig. 3). In the preliminary examination, reduction of cytochrome *c* in

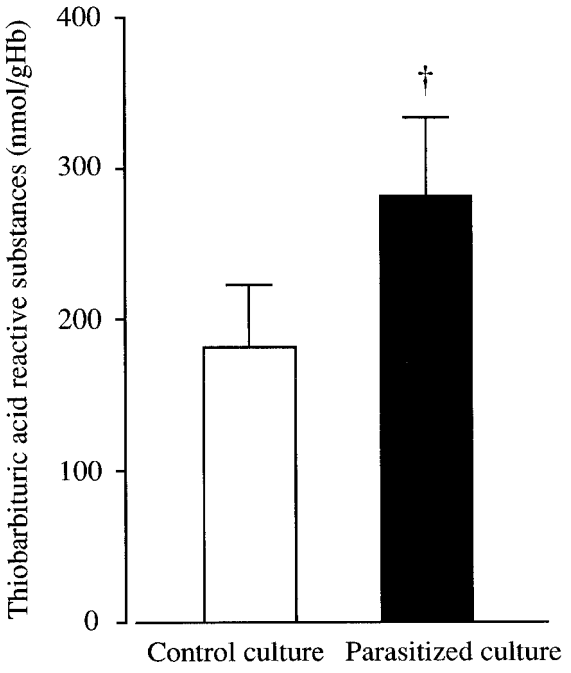

Fig. 1. Thiobarbituric acid reactive substances in erythrocytes from control (open column) and parasitized cultures (closed column) on Day 7 after the onset of culture. Vertical bars indicate mean ± standard deviation of six experiments. † $p<0.005$, compared with the value obtained in control culture by means of Student's *t*-test. Hb: hemoglobin.

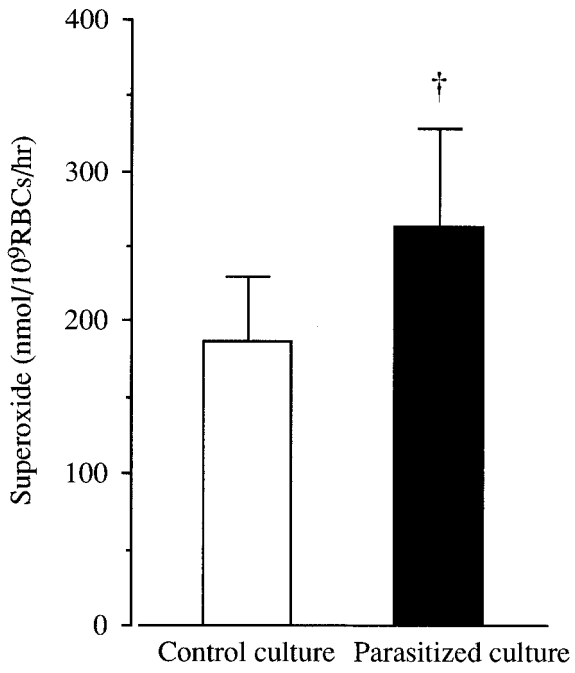

Fig. 2. Superoxide generation rate in control (open column) and parasitized cultures (closed column) on Day 7 after the onset of culture. Vertical bars indicate mean ± standard deviation of nine experiments. † $p<0.005$, compared with the value obtained in control culture by means of Student's *t*-test. RBC: erythrocyte.

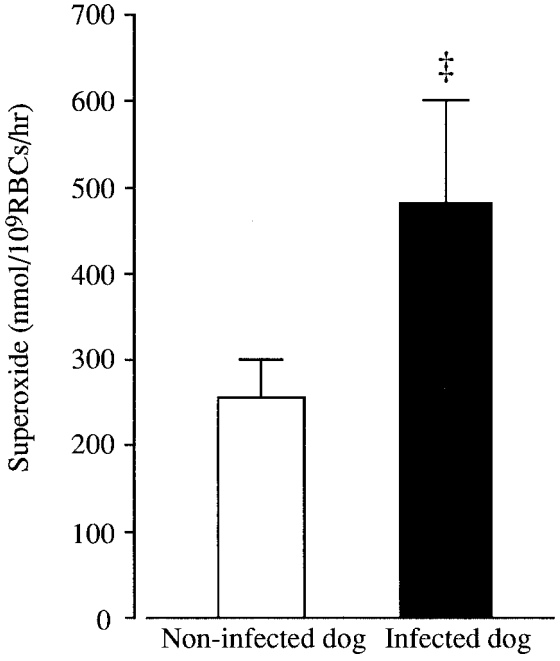

Fig. 3. Superoxide generation rate in erythrocytes collected from non-infected dogs (open column) and dogs infected with *Babesia gibsoni* (closed column). Vertical bars indicate mean ± standard deviation of thirteen experiments. ‡ $p<0.001$, compared with the value obtained in non-infected dogs by means of Student's *t*-test. RBC: erythrocyte.

blood of infected dogs was completely inhibited by the addition of 10 μM superoxide dismutase (data not shown).

The activities of erythrocytic antioxidative enzymes were higher in *B. gibsoni*-infected dogs than in non-infected dogs (Table 1).

In *B. gibsoni*-infected dogs, there was a significant positive correlation between the amount of superoxide generation and the number of reticulocytes in their peripheral blood ($r=0.631$, $p<0.05$) (Fig. 4A). Reticulocyte percentage of *B. gibsoni*-infected dogs varied from 2.4% to 28.3% during the experimental periods. The rate of superoxide generation in reticulocyte suspensions prepared from phlebotomized non-infected dogs increased in proportion to the increase of the percentage of reticulocytes in the suspensions ($r=0.927$, $p<0.001$) (Fig. 4B). The superoxide generation in the blood of *B. gibsoni*-infected dogs was higher than that in non-infected blood possessing the similar reticulocyte count to the infected blood (Fig. 4).

Table 1. Activities of erythrocyte antioxidative enzymes in non-infected dogs and dogs infected with *Babesia gibsoni*

Antioxidative enzymes	Non-infected dogs	Infected dogs
Superoxide dismutase ($\times 10^3$ U/gHb)	4.59 ± 0.79	6.83 ± 1.21 ‡
Catalase ($\times 10^3$ IU/gHb)	0.76 ± 0.58	0.94 ± 0.52
Glutathione peroxidase (IU/gHb)	179.9 ± 37.3	186.9 ± 44.3
Glutathione reductase (IU/gHb)	4.91 ± 2.77	8.89 ± 3.03 *
Glucose-6-phosphate dehydrogenase (IU/gHb)	7.31 ± 2.44	14.59 ± 2.72 ‡
6-phosphogluconate dehydrogenase (IU/gHb)	1.84 ± 1.61	2.45 ± 1.28
Glutathione S-transferase (IU/gHb)	0.45 ± 0.40	2.76 ± 0.67 ‡
Methemoglobin reductase (IU/gHb)	9.21 ± 1.47	9.47 ± 1.50
Reticulocyte count (%)	0.6 ± 0.3	9.0 ± 7.7 †

Values are expressed as mean ± standard deviation of nine experiments. * $p<0.01$, † $p<0.005$ and ‡ $p<0.001$, compared with the values obtained for non-infected dogs by means of Student's *t*-test. Hb: hemoglobin.

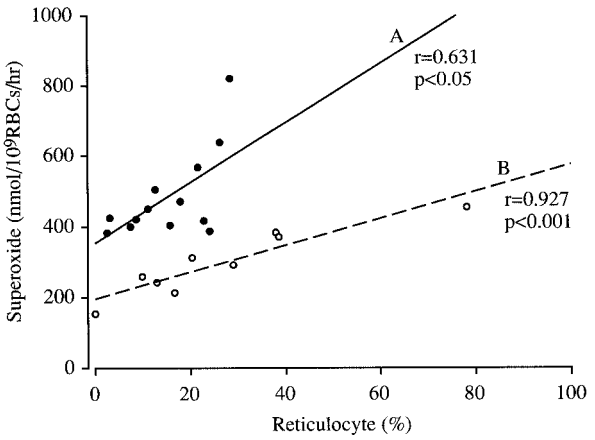

Fig. 4. Correlation between superoxide generation rate and reticulocyte percentage. Closed circles and the solid line (A) represent data obtained from dogs infected with *Babesia gibsoni*. Open circles and the broken line (B) represent data obtained from phlebotomized dogs. Correlation coefficient (r) and level of significance were obtained by means of Pearson product moment linear regression analysis.

DISCUSSION

We previously hypothesized that active oxygen would be generated by the multiplication of *B. gibsoni* [10], and the present study showed a significant increase of superoxide generation in erythrocytes cultured with *B. gibsoni* parasites (Fig. 2). In addition, those cells cultured with the parasite had significantly higher TBARS in their membranes than erythrocytes cultured without parasites (Fig. 1), indicating that lipid peroxidation was greater in the infected cells than in non-infected erythrocytes. The observations indicate that superoxide is produced by the parasite itself or the parasitized erythrocytes, and suggest that oxidative damage might be done to the host erythrocytes by the parasites.

Similarly, superoxide generation was also higher in erythrocytes of *B. gibsoni*-infected dogs than in non-infected dogs (Fig. 3). Although the mechanism of the increased generation of superoxide in infected erythrocytes is presently unknown, it is likely that an antioxidant protective mechanism of host erythrocytes might be impaired by the parasite. For example, it has been reported that murine erythrocytes infected with the malaria parasite (*Plasmodium berghei*) tend to become quantitatively deficient in the activity of superoxide dismutase, the enzyme responsible for catalyzing the dismutation of superoxide to hydrogen peroxide [5, 6]. In addition, an increase in parasite load is accompanied by decreased activities of the antioxidative enzymes catalase [6, 7, 11, 13, 17], glutathione peroxidase [17, 20], glucose-6-phosphate dehydrogenase [14] and methemoglobin reductase [16, 17] in erythrocytes infected with the malaria parasite. In the present study, however, the activities of those enzymes in erythrocytes of infected dogs were higher than those of non-infected dogs (Table 1). This result seemed to be due to the high percentage of reticulocytes in infected dogs because the activities of enzymes are higher in reticulocytes than in mature erythrocytes [15, 18]. Moreover, the generation of superoxide in reticulocyte suspensions was increased in proportion to the increase in the number of reticulocytes in the suspensions, indicating that reticulocytes can produce a higher amount of superoxide than mature cells in spite of high activities of antioxidative enzymes. Reticulocytes contain ribosomes and mitochondria in the cytoplasm and consume much oxygen to maintain their metabolism. In addition, a large amount of superoxide anions is produced in mitochondria when adenosine triphosphate (ATP) is synthesized via the pathway of the electron transmission system. Thus, it is reasonable to suppose that reticulocytes are able to produce a large amount of superoxide compared to mature erythrocytes. However, superoxide generation in the blood samples from infected dogs was much higher than that in reticulocyte suspensions from normal dogs even with the same percentage of reticulocytes in both samples (Fig. 4). From these findings, we hypothesized that the increase of superoxide generation in the blood of *B. gibsoni*-infected dogs was also induced by *B. gibsoni* infection, and that lipid peroxidation occurs in erythrocyte membrane, though the mechanism of this phenomenon remains to be clarified.

To our knowledge, there is no direct evidence of generation of active oxygen species by *Babesia* parasites. In malaria parasites, direct oxidation of erythrocytes by malaria infection has been reported [8]. Erythrocytes parasitized with *P. falciparum* [20], *P. vinckei* [2], and *P. berghei* [11] exhibited increased lipid peroxidation. These observations have been taken as indirect evidence for oxidant production by the parasites. However, the nature and origins of the reactive oxygen species responsible for enhanced oxidation of host erythrocytes have not been elucidated.

In conclusion, the present study demonstrated that superoxide anions are produced in *B. gibsoni*-infected erythrocytes *in vitro*, which suggests that the parasite causes measurable oxidation of the host erythrocytes during its multiplication in the cells.

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