Reactivity of Serum Anti-Erythrocyte Membrane Antibody in *Babesia gibsoni*-Infected Dogs

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ABSTRACT. Reactivity of anti-erythrocyte membrane (RBCM) antibody in sera of *B. gibsoni*-infected dogs on native RBCM and erythrocyte surface (RBCs) was examined using an enzyme linked immunosorbent assay. Anti-RBCM antibody attached to native RBCM and the surface of erythrocytes (RBC) treated with phenylhydrazine or neumaminidase, but not to the surface of non-treated RBC. Based on these results, anti-cytoskeletal protein antibody in sera of *B. gibsoni*-infected dogs is considered to be specific to native RBCM, and furthermore free radical-induced oxidative stress imposed on RBC and sialic acid removal from glycoproteins of RBC are considered necessary for anti-transmembrane protein antibody in sera of *B. gibsoni*-infected dogs to be bound to RBCs. These results are important to elucidating the mechanism of the marked increase in RBCs-bound IgG value and anemia in *B. gibsoni* infection.

Key words: anemia, anti-erythrocyte membrane antibody, *B. gibsoni* infection.


*Babesia gibsoni*, a causative agent of canine babesiosis, is a tick-transmitted hemoparasitic protozoan and the intraerythrocytic stages or piroplasms account for the primary clinical findings of peracute, acute or chronic anemia. Regarding the mechanism of the anemia, excessive hemolysis has been explained on the basis of a direct toxic effect of hemolytic factor(s) on the erythrocytes (RBC) by Onishi et al. [14]. Murase and Maeda [11] have reported on the basis of a cellular immunologic mechanism that erythrophagocytic activity of macrophages was enhanced in *B. gibsoni* infection. Our previous studies demonstrated that anti-erythrocyte membrane (RBCM) antibody levels in sera [1, 2] and erythrocyte surface (RBCs)-bound IgG values [3] were raised in *B. gibsoni*-infected dogs, suggesting that antibody-mediated anemia might occur in *B. gibsoni* infection. However a humoral immunologic mechanism has been considered to be unresponsible for anemia in *B. gibsoni* infection, it being also suspected from the results of our preliminary experiment that anti-RBCM antibody levels remained unchanged in sera of *B. gibsoni*-infected dogs even after absorbed with self or non-self RBCs. Considering raised RBCs-bound IgG values, the binding of anti-RBCM antibody to RBCs seems to require some changes in RBCM, as documented in aged or senescent RBC [9]. Senescent RBC are recognized and phagocyctized by macrophages on the basis of the specific binding of anti-RBCM IgG antibody to senescent erythrocyte antigen generated by oxidation and removal of sialic acid [9]. In malaria, RBC were shown to be under oxidative stress above defensive ability of scavenger in RBCM [5, 7], and to accumulate much more malonyldialdehyde (MDA), an index of lipid peroxidation [6]. Free radical is known to be released by activated macrophages [12, 13]. From these results, it is hypothesized that erythrocyte oxidative stress imposed by activated macrophages damages the RBCM in *B. gibsoni* infection, and thereby anti-RBCM antibody is bound to the damaged RBCs which are then recognized and phagocyctized by macrophages.

In this paper, we report on the reactivity of serum anti-RBCM antibody in *B. gibsoni*-infected dogs and discuss a possible immunologic mechanism of anemia in *B. gibsoni* infection.

*B. gibsoni* parasites used were originally isolated from a dog at the Veterinary Teaching Hospital, Miyazaki University in Japan [2]. Using an enzyme linked immunosorbent assay (ELISA) [1], sera of three dogs intravenously inoculated with approximately 3 × 10⁶ parasitized RBC were examined for reactivity with self RBCM, and the surface of treated and non-treated RBC. Prior to experimental infection, RBCM was prepared according to the method of Tomoda et al. [17] and stored at −70°C until use. A 2.5% suspension of RBC in RPMI-1640 (Nissui, Tokyo, Japan), obtained from the inoculated dogs recovered from clinical infection, was incubated with phenylhydrazine (0.1 mM) or neumaminidase from *Clostridium perfringens* (SIGMA Chemical Company, ST. Louis, U.S.A.) (0.125 IU/mL) for 60 min in an agitating water at 37°C. Non-treated RBCM were also incubated in the same manner. The treated and non-treated RBCM and RBCM suspended, 2:1 (v/v) and 1:1 (v/v), respectively, in autologous sera of the infected dogs, were incubated for 60 min in an agitating water at 37°C. At the same time sera of the infected dogs were incubated without either RBCM or RBCM in the same manner. After centrifuging the resultant medium at 400 × g for 5 min, sera were collected and examined for anti-RBCM ELISA level.

Compared to anti-RBCM ELISA levels of infected dog sera incubated without RBCM (A), those of the sera incubated with non-treated self RBCM (B) increased slightly (no statistical significance), while those of the sera incubated with treated self RBCM (C, D) reduced markedly (Fig. 1).

As shown in Fig. 2, infected dog sera incubated with self RBCM (B) showed prominently reduced anti-RBCM ELISA levels, compared to those of the sera incubated without RBCM.

Anti-RBCM antibody in sera of infected dogs reacted with native RBCM and degraded RBCs, but not with native RBCs. From these results, anti-cytoskeletal protein antibody is considered to be specific to native RBCM, while anti-transmembrane structural protein antibody to attach only to denatured RBCs. The same affinity has been reported of native anti-cytoskeletal [10] and transmem-
brane [9] protein antibodies for RBCm in humans. These similarities in antibody affinity indicate that anti-RBCm antibody induced by B. gibsoni infection may be the same as produced physiologically.

In spite of the increase in RBCs-bound IgG value in B. gibsoni-infected dogs [3], no anti-RBCm antibody in sera attached to self RBCs of the dogs recovered from clinical infection, this indicating that in order to bind anti-RBCm antibody to RBCs some changes may be required in RBCm, such as the generation of senescent erythrocyte antigen. Such changes as oxidation and sialic acid removal in RBCm are essential to antibody-mediated clearance of senescent RBC [9], this prompting us to treat RBC with phenylhydrazine or neuraminidase. As a result, in sera of B. gibsoni-infected dogs anti-RBCm antibody was found bound to the surface of RBC undergoing free radical-induced oxidation and sialic acid removal, as in the physiological removal of senescent RBC.

Activated macrophages are documented to release parasitidal free radical [13]. Macrophages are known to be activated in B. gibsoni infection [11] and RBC pass through sinusoids combined with a large number of macrophages slowly and for more extended time than normal because of splenomegaly, so RBCm may be damaged by free radical, and thereby antigens immunoreactive to anti-RBCm antibody may be generated and MDA be elevated. Increased MDA deprives RBC of deformability [15].

In B. gibsoni infection, activated macrophages may impose oxidative stress on RBC, and thereby the RBC may be coated with innate anti-RBCm antibody produced more markedly than normal, accumulate MDA and become vulnerable to phagocytosis by macrophages. Sialic acid removal, important in clearing senescent RBC [4, 8, 9], may also account for anti-RBCm antibody to be bound to RBCs in B. gibsoni infection. Transient autoimmune hemolytic anemia sometimes developed in the infection with such viruses as contain neuraminidase [8]. However it remains unclear whether in B. gibsoni infection oxidative stress induces the same sialic acid removal as catalyzed by neuraminidase, and whether B. gibsoni parasites contain neuraminidase.

In malaria, oxidative stress has been documented overcome by anti-malarial drug therapy [16], presumably this explaining the discrepant result of our previous study [2] that recovery rate from anemia was more than that expected from the elevated anti-RBCm ELISA levels after anti-babesial drug therapy; that is, RBCm may have been no longer changed antigenically because of disappearance of oxidative stress imposed on RBC, and accordingly it may not have allowed anti-RBCm antibody to be bound to RBCs. Consequently macrophages may not recognize RBC.

Our study is now under way to clarify the process of oxidative damages to RBC and the origin of free radical in B. gibsoni infection.

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REFERENCES