Elevated Erythrocyte-Bound IgG Value in Dogs with Clinical *Babesia gibsoni* Infection

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ABSTRACT. Erythrocyte (RBC)-bound IgG values (IgG concentration, *ng*/m*l*) were examined in 8 *Babesia gibsoni*-infected and 10 healthy dogs by a competitive enzyme immunoassay. Five dogs with clinical *B. gibsoni* infection, manifesting severe anemia, were observed to have increased RBC-bound IgG values compared to dogs with subclinical *B. gibsoni* infection and healthy controls, this suggesting that anemia in *B. gibsoni* infection may be explained in part on the basis of a humoral immunologic mechanism.—KEY WORDS: anemia, *Babesia gibsoni*, erythrocyte-bound IgG.

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Babesia gibsoni, a causative agent of canine babesiosis, is tick-transmitted hemoparasitic protozoa and presence of intraerythrocytic stages or piroplasms causes peracute, acute, or chronic anemia as the primary clinical findings. Regarding the mechanism of anemia, excessive hemolysis has been explained on the basis of a direct toxic effect of hemolytic factors on the erythrocytes (RBCs) by Onishi *et al.* [11]. Murase and Maede [10] have reported on the basis of a cellular immunologic mechanism that erythrophagocytic activity of macrophages was increased in *B. gibsoni* infection. We suggested that a humoral immunologic mechanism might be involved in anemia [1, 2]. Our previous study has demonstrated that anti-red blood cell (RBC) membrane ELISA levels were elevated in sera of *B. gibsoni*-infected dogs. This result raises a possibility that the elevated production of autoantibodies to skeletal proteins of RBCs [9] may have been triggered by the acceleration of RBC destruction, thereby resulting in the increased anti-RBC membrane ELISA levels in sera of *B. gibsoni*-infected dogs. Anti-cyto-skeleton autoantibodies are never bound to intact RBCs, nor do they contribute to the immune-mediated anemia. In order to examine the degree to which a humoral immunologic mechanism may account for anemia in *B. gibsoni* infection, our present study was designed to measure RBC-bound IgG in *B. gibsoni*-infected dogs by a competitive enzyme immunoassay (EIA).

Eight *B. gibsoni*-infected dogs, examined at the Veterinary Teaching Hospital, Miyazaki University in Japan, were used in the present study. *B. gibsoni*-infected dogs were divided into subclinically and clinically infected dog groups to examine the correlation between packed cell volume and RBC-bound IgG value in *B. gibsoni* infection. Three dogs (cases 1 to 3) were subclinically infected with *B. gibsoni* and the other 5 dogs (cases 4 to 8) suffered from clinical infection. Ten healthy dogs constituted the control group. All dogs had no history of receiving blood transfusion. The direct antiglobulin tests were performed in *B. gibsoni*-infected dogs by standard techniques. Parasitemia level was examined with Wright's-Giemsa-stained blood smears. Anti-parasite antibody levels in sera of subjected dogs were measured by ELISA. The parasite antigens were prepared by the previously reported method [4]. By using sera of 20 healthy dogs (courteously provided by Laboratory Animal Science and Toxicology Laboratories, Sankyo Co., Ltd., Shizuoka, Japan), an upper limit of normal values was determined to be 0.22 (optical density at 490 nm).

RBC-bound IgG was quantitatively determined by a competitive EIA [3]. Heparinized blood samples collected from dogs were subjected to dextran sedimentation method [5]. A 25% RBC suspension in cold phosphate buffered saline (PBS), pH 7.4, was applied to cellulose powder (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) column to remove leukocytes. The eluted RBC suspension was centrifuged at 600 G for 10 min. Packed RBCs were resuspended in PBS and washed five times by centrifugation at 600 G for 5 min. The resultant packed RBCs free of plasma IgG and leukocytes were subjected to a competitive EIA. A microplate (Falcon 3912, Becton Dickinson, CA, U.S.A.) was coated with 0.05 m*l* of dog IgG (Cappel, PA, U.S.A.) (1.25 μg/m*l*) diluted in 0.05 M carbonate buffer (pH 9.6), and incubated overnight at 4°C. After washing the plate with PBS containing 0.1% bovine serum albumin (BSA), 0.2 m*l* of PBS containing 1% BSA was added to the plate, and the plate was incubated at room temperature for 2 hr. Fifty μ*l* of a 12% RBC suspension of subjected dogs was preincubated at 37°C for 30 min in a glass tube with an equal volume of peroxidase-conjugated anti-dog IgG (Rockland, PA, U.S.A.) diluted 1:10,000 with 0.1% BSA-PBS, and the glass tube was shaken every 10 min. After blocking procedure, the plate was washed with 0.1% BSA-PBS. One hundred μ*l* of preincubated mixture in a glass tube (subjected RBC suspension with the enzyme-conjugated anti-dog IgG) was added to the plate. Thereafter the plate was incubated at 37°C for 30 min and shaken every 10 min. To form a standard curve, 50 μ*l* of known amounts of serial dilutions of dog IgG (Cappel, PA, U.S.A.) was preincubated at 37°C for 30 min in a glass tube with an equal volume of peroxidase-conjugated anti-dog IgG, and then added to the dog IgG-coated plate. Thereafter the plate was incubated at 37°C for 30 min and shaken every 10 min. After the plate was washed five times with PBS containing 0.05% Tween 20, 0.1 m*l* of o-phenylenediamine dihydrochloride (Nacalai Tesque, Inc., Kyoto, Japan) at a concentration of 4 mg/m*l* in enzyme substrate buffer (0.1 M citric acid, 0.2 M disodium hydrogen phosphate and 0.012% hydrogen peroxide) was added to each well. The plate was incubated at room temperature for 20 min. The colorimetric

reaction was terminated by adding 0.05 m*l* of 2 M sulfuric acid to each well. Thereafter the well contents were measured for absorbance at 490 nm with a micro ELISA spectrophotometer. RBC-bound IgG value base line was estimated by using heterologous system consisting of intact RBC suspension of dogs in normal and pathologic states characterized by anemia, rabbit IgG (Zymed Laboratories, Inc., CA, U.S.A.) and peroxidase-conjugated anti-rabbit IgG (Tago, Inc., CA, U.S.A.). RBC-bound IgG value, expressed as IgG concentration (*ng*/m*l*), was calculated as follows: RBC-bound IgG value estimated based on the standard curve A — RBC-bound IgG value base line estimated based on the standard curve B. The standard curves A and B were obtained by using serially diluted dog IgG and rabbit IgG in 0.1% BSA-PBS, respectively.

As shown in Table 1, anti-parasite ELISA levels of 10 healthy dogs were below an upper negative limit of 0.22, consistent with no parasitemia. The higher anti-parasite ELISA levels in *B. gibsoni*-infected dogs were compatible with parasitemia. RBC-bound IgG value base line was estimated to be 7.5 *ng*/m*l* by using heterologous system. RBC-bound IgG values (IgG concentration, *ng*/m*l*), of 10 healthy dogs ranged from 6.5 to 20 with a mean value of 13.5 ± 4.6 (SD). Mean ± 2SD (range 4.3 to 22.7) was considered to be a normal range. The direct antiglobulin test was negative in 3 dogs (cases 1 to 3) with subclinical *B. gibsoni* infection, and RBC-bound IgG values were 17, 10 and 6.5, respectively, within the normal range. Packed cell volumes (PCVs) in three dogs with subclinical *B. gibsoni* infection were within the normal range. On the other hand, 5 dogs (cases 4 to 8) with clinical *B. gibsoni* infection demonstrated elevated RBC-bound IgG values of 38, 38, 34, 83 and 126, respectively, above an upper limit of the normal of 22.7, and the direct antiglobulin test was positive in five dogs. Five dogs with clinical *B. gibsoni* infection developed severe anemia, as indicated by extremely low PCVs.

If RBC-bound IgG values of subjected dogs were expressed as the number of IgG molecules per RBC, RBC-bound IgG values of 10 healthy dogs ranged from 12 to 63 IgG molecules per RBC, those values being similar to the normal range in humans [6, 7]. RBC-bound IgG values of 5 clinically infected dogs were 105, 106, 93, 230 and 350, respectively. These values are consistent with the results of Coombs' test because the critical positive range of Coombs' test is 80 to 120 IgG molecules per RBC [13]. Furthermore similar RBC-bound IgG values (73 to 120 in patients with Evans syndrome, more than 142 IgG molecules per RBC in those with autoimmune hemolytic anemia) were reported in humans manifesting immune-mediated anemia [13]. In the present assay, IgG solution in 0.1% BSA-PBS, not RBC-bound IgG, was employed to form a standard curve for the determination of RBC-bound IgG value, so the number of IgG molecules per RBC cannot be determined exactly. However relative values of RBC-bound IgG of dogs manifesting severe anemia can be determined as primary clinical finding in *B. gibsoni* infection compared to those of healthy dogs.

As well as RBCs of healthy humans, those of healthy dogs were found to be coated by some IgG antibodies in that RBC-bound IgG values were above the basal. IgG antibody bound onto the RBC membrane in the physiologically normal state was documented to opsonize senescent RBCs to promote phagocytosis of senescent RBCs by macrophages [8]. That is, RBC membrane-binding IgG in

Table 1. RBC-bound IgG value measured by a competitive enzyme immunoassay

Dog	Coombs' test[a)]	PCV (%)	Parasitemia(%)	Anti-parasite ELISA level[b)]	RBC-bound IgG value[c)]
Healthy dogs (n=10)	NT	40<	0	*	6.5 to 20 (13.5±4.6[d)])
Dogs with subclinical *B. gibsoni* infection					
Case 1	N	40<	0.1≧	0.77	17
Case 2	N	40<	0.1≧	1.38	10
Case 3	N	40<	0.1≧	3.52	6.5
Dogs with clinical *B. gibsoni* infection					
Case 4	P	12	1.0	2.57	38
Case 5	P	10	7.5	0.79	38
Case 6	P	15	2.2	0.86	34
Case 7	P	15	2.5	1.00	83
Case 8	P	14	5.5	1.55	126

a) NT: Not tested, P: Positive, N: Negative.
b) Anti-parasite ELISA level is expressed as optical density at 490 nm. *=ELISA levels below an upper negative limit of 0.22 are considered to be normal.
c) RBC-bound IgG value, expressed as IgG concentration (*ng*/m*l*), is calculated as follows: RBC-bound IgG value measured based on the standard curve A–7.5 *ng*/m*l* (RBC-bound IgG value base line measured with the standard curve B). The standard curves A and B were obtained by using serially diluted dog IgG and rabbit IgG in 0.1% BSA-PBS, respectively.
d) Mean±SD.

the physiologically normal state is considered to play a physiologic and homeostatic role in clearance of senescent RBCs in collaboration with macrophages.

The dogs developing clinical *B. gibsoni* infection with severe anemia were observed to have increased RBC-bound IgG values compared to dogs with subclinical *B. gibsoni* infection and healthy controls, this suggesting that not only antibody to skeletal proteins of RBCs but that to surface proteins exposed to immune system may be more markedly produced in clinically *B. gibsoni*-infected dogs compared to those in the physiologically normal state. However immune complex (IC) consisting of parasite antigens, anti-parasite antibody and C3b may account for elevated RBC-bound IgG values of clinically *B. gibsoni*-infected dogs in that RBC membranes have C3b receptor. The binding of anti-RBC IgG antibody and IC onto RBC membranes may be responsible for immune-mediated anemia. Coupled with increased erythrophagocytic activity of macrophages and splenomegaly in *B. gibsoni* infection, elevated RBC-bound IgG value is likely to account in part for acceleration of RBC destruction. However, besides RBC-bound IgG value, the arrangement of IgG molecules on RBC membrane is important for RBC recognition by macrophages [12]. To examine significance of increased RBC-bound IgG value and immunologic difference in RBC-bound IgG between *B. gibsoni* infections and normal cases, further detailed studies are necessary.

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