Anti-Erythrocyte Membrane Antibodies Detected in Sera of Dogs Naturally Infected with *Babesia gibsoni*

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ABSTRACT. Due to the potential for anti-erythrocyte membrane antibodies as possible enhancers of erythrocyte destruction, the presence of serum anti-erythrocyte membrane antibodies in 31 dogs with *Babesia gibsoni* infection admitted to a veterinary hospital was investigated by an enzyme linked immunosorbent assay (ELISA) and immunoblotting analyses. This infection resulted in an increase of anti-erythrocyte membrane antibodies in 84% (IgG) and 74% (IgM) of 31 infected dogs, respectively. This was confirmed by the similarity in the protein profiles of the erythrocyte membrane antigens immunoblotted with rabbit antiserum to dog erythrocyte membrane antigens and infected dog serum. These results suggest the production of anti-erythrocyte membrane antibodies was induced by *B. gibsoni* infection.—KEY WORDS: anti-erythrocyte membrane antibody, *Babesia gibsoni*, dog, ELISA, immunoblotting.

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Babesia gibsoni is a causative agent of canine babesiosis, and its intraerythrocytic stages induce anemia [7]. The mechanism of anemia still remains to be elucidated. It was revealed that autoimmune responses occur in the progress of *Babesia* and *Plasmodium* infections [1, 9, 11, 13, 14, 17], thereby resulting in the production of anti-erythrocyte membrane antibodies [9, 11]. The suggestion was made that in the course of *P. berghei* and *B. rodhaini* infections the elevated frequency of erythrocyte destruction is in part due to phagocytosis of erythrocytes opsonized with anti-erythrocyte membrane antibodies by the mononuclear phagocytes [9]. The presence of anti-erythrocyte membrane antibodies in dogs infected with *B. gibsoni* has not yet been confirmed, nor has it been demonstrated whether anti-erythrocyte membrane antibodies accelerate the erythrocyte destruction in *B. gibsoni* infection. To elucidate the mechanism of anemia in *B. gibsoni* infection, the present study was designed to investigate the presence of anti-erythrocyte membrane antibodies in the sera of dogs spontaneously infected with *B. gibsoni*, using ELISA and immunoblot.

MATERIALS AND METHODS

Clinical samples: Serum samples were obtained from both dogs with spontaneous *B. gibsoni* infection confined at the Veterinary Teaching Hospital, Miyazaki University in Japan, and normal healthy ones (Laboratory Animal Science and Toxicology Laboratories, Sankyo Co., Ltd.). None of the dogs had a history of receiving blood transfusions and administration. The sera were stored at −70°C until use.

Preparation of the erythrocyte membrane antigens: Blood was collected from a non-infected dog. Following dextran sedimentation [2], erythrocytes were applied to CF-cellulose powder (Sigma) column to remove leukocytes. White ghost membrane was prepared from leukocyte-free erythrocytes according to the method of Tomoda *et al.* [15]. The protein concentration of white ghost membrane prepared was determined. The preparation was stored at −70°C until use.

ELISA procedure: ELISA was performed by a modification of the method of Ishikawa *et al.* [6]. Microplate (Falcon 3912, Becton Dickinson, CA, U.S.A.) was coated with 0.05 m*l* of the erythrocyte membrane antigens (20 μg protein/m*l*) diluted in 0.05 M carbonate buffer (pH 9.6), and incubated for 12 hr at 4°C. After the washing of the plate with PBS, 0.1 m*l* of PBS containing 2% bovine serum albumin (BSA) was added to each well, and incubated at 37°C for 1 hr. After washing as described above, 0.05 m*l* of the test serum diluted 1:100 with PBS containing 0.05% Tween 20 and 0.1% BSA was added to each well, and incubated at 37°C for 30 min. After washing again, each well was filled with 0.05 m*l* of peroxidase-conjugated rabbit anti-dog immunoglobulins (IgG, IgM) (Heavy & light chain specific, Cappel, PA, U.S.A.) diluted

1:1000 with the same buffer as used for the test serum, and incubated at 37°C for 30 min. This was washed again and 0.1 m*l* of o-phenylenediamine dihydrochloride (Nacalai Tesque, Inc., Kyoto, Japan) at a concentration of 0.4 mg/m*l* in enzyme substrate buffer (0.1 M citric acid, 0.2 M di-sodium hydrogen phosphate and 0.012% hydrogen peroxide) was added to each well, and the plate was incubated at room temperature for 30 min. The colorimetric reaction was terminated by adding 0.05 m*l* of 2 M sulfuric acid to each well, after which the absorbance of the well contents was measured at 492 nm with a micro-ELISA spectrophotometer (Easy Reader, EAR 400 FW, SLT Co., Austria).

Preparation of antiserum: For Western immunoblots, antiserum to dog erythrocyte membrane antigens was prepared using a rabbit weighing ca. 2 kg. As described above, the sample (10 mg protein/m*l*) of the erythrocyte membrane antigens from a non-infected dog was prepared. Four m*l* of an emulsion consisting of the sample and complete Freund adjuvant (Difco) was administered subcutaneously in multiple sites on the back of the animal. This animal was reimmunized by intravenous administration of 10 m*l* of the suspension composed of the sample and physiological saline. Blood collection was carried out on the 10th day after the booster immunization. Antiserum titer was evaluated by ELISA and agglutination of erythrocytes before storage at −70°C.

SDS-PAGE and Western immunoblots: A 50 μl portion of the erythrocyte membrane antigens (1 mg protein/m*l*) boiled in SDS sample buffer [10] was electrophoresed in a slab gel with a 4.5% stacking gel and a 10% resolving gel using the discontinuous buffer system [10]. According to the methods of Towbin *et al.* [16], proteins separated by SDS-PAGE were transferred electrophoretically to nitrocellulose paper. The nitrocellulose paper was washed 3 times in 10 mM Tris- buffered saline (TBS), pH 7.4, and incubated in TBS containing 3% bovine serum albumin (BSA) for 1 hr at 37°C. Test serum diluted 1:100 in TBS containing 3% BSA was incubated with the paper for 30 min at room temperature. The sheet, washed two times for 10 min in TBS containing 0.05% Nonidet P-40 (NP-40) (Sigma), was mixed with peroxidase-conjugated rabbit anti-dog or goat anti-rabbit immunoglobulins (IgG fraction) (Heavy & light chain specific, Cappel, PA, U.S.A.) diluted 1:1,000 in TBS containing 3% BSA. Following incubation at room temperature for 30 min, the sheet was washed three times for 30 min in TBS containing 0.05% NP-40. The sheet was developed in a solution of 0.025% 3,3'-diaminobenzidine tetrahydrochloride, 0.015% hydrogen peroxide and 0.05M Tris-HCl (pH 7.6). Colorimetric reaction was terminated by exhaustive rinsing in deionized water.

Statistics: The statistical significance of data was analyzed by student's *t*-test.

RESULTS

Detection of anti-erythrocyte membrane IgG antibodies: Sera from thirty-one dogs infected with *B. gibsoni* and twenty-four normal controls were screened for the presence of anti-erythrocyte membrane IgG antibodies by ELISA. There was a wide range of ELISA levels (optical density) exhibited by the individual sera, ranging from 0.023 to 0.597 (Fig. 1). As shown in Table 1, the mean ELISA level of infected dogs was 0.199 ± 0.118 (S.D.) and this was significantly higher than that of healthy controls, 0.052 ± 0.017 (S.D.) giving an upper limit of normal of 0.086 ($p<0.001$). Of the infected subjects, 84% gave positive reactions.

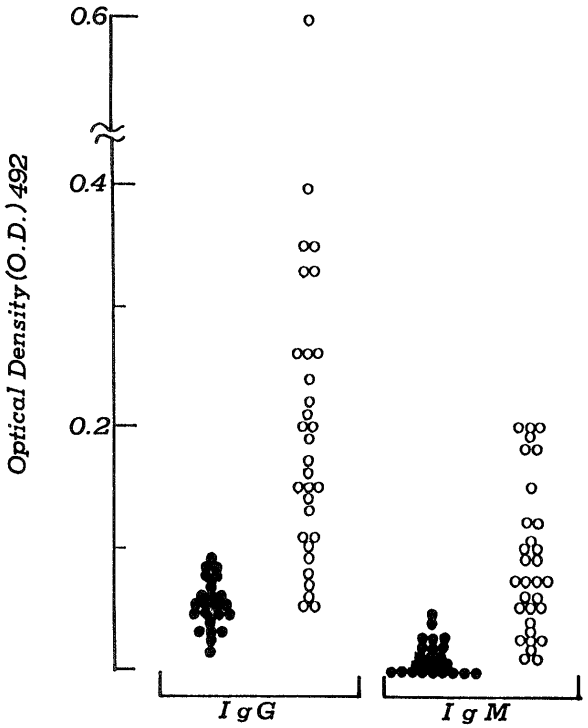

Fig. 1. Reactivity in ELISA of individual serum in infected dogs (○) and normal controls (●) to the erythrocyte membrane antigens.

Detection of anti-erythrocyte membrane IgM antibodies: As in the case of IgG, all sera of both infected and normal subjects were examined for the presence of anti-erythrocyte membrane IgM antibodies. Subjects ranged in ELISA level from 0 to 0.208 with the maximum level of 0.208 similar to the mean IgG ELISA level of infected dogs (Fig. 1). From the upper limit of normal of 0.04 given by the mean ELISA level of 0.012 ± 0.014 (S.D.) in healthy dogs, twenty-three out of 31 (74%) infected dogs showed positive reactions. The mean level of 0.090 ± 0.049 (S.D.) in infected dogs was significantly higher than that in normal controls ($p<0.001$), as shown in Table 1.

Western blot: The erythrocyte membrane antigens were transferred to nitrocellulose paper and immunoblotted with sera from normal dogs (NDS; lane A), infected dogs (IDS; lane B), immune rabbits (IRS; lane C), and normal rabbits (NRS; lane D), to detect anti-erythrocyte membrane IgG antibodies in sera of infected dogs, and to compare the components reacting with IDS and IRS. As shown in Fig. 2, antibodies reacting with several components of the erythrocyte membrane antigens were recognized not only in IRS but likewise in IDS. The molecular weights of the components reacting with both IRS and IDS were 90,000, 65,000, 63,000, 60,000, 55,000, 50,000 48,000, 46,000, 33,000 and 30,000 daltons. However, the intensity of reaction between IDS and a component with molecular weight of 48,000 daltons was the strongest. Only components with molecular weights of 61,000 and 42,000 daltons reacted with IDS alone. No components reacted with either NDS or NRS.

DISCUSSION

For clarifying antibody-mediated erythrocyte destruction, we examined anti-erythrocyte membrane antibodies in sera from dogs infected with *B. gibsoni*.

Table 1. Anti-erythrocyte membrane ELISA levels in sera of dogs infected with *B. gibsoni*

Sera	IgG	IgM
Normal controls (24)[a)]	0.052±0.017[b)]	0.012±0.014[b)]
Infected dogs (31)	0.199±0.118*	0.090±0.049*

a) Number of individual sera examined in parentheses.
b) O.D. value at 492 nm.
*: $P<0.001$.
Data represent mean±S.D.

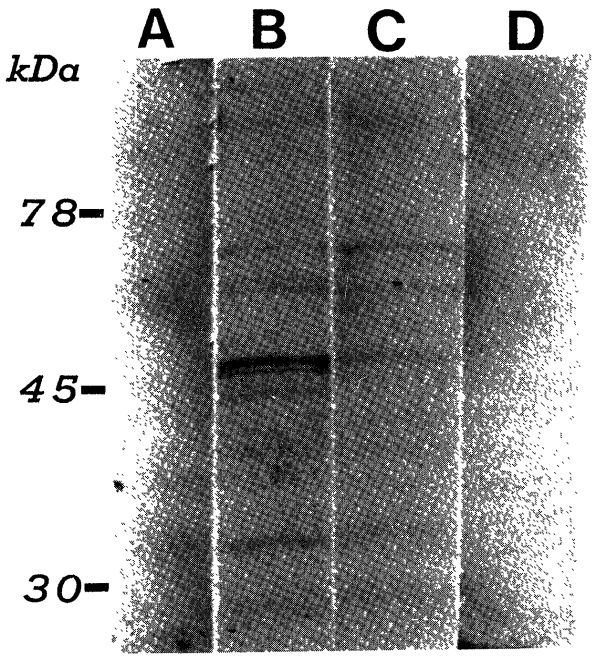

Fig. 2. Comparison of the protein profiles of the erythrocyte membrane antigens immunoblotted with infected dog and immune rabbit serum. Lane A, normal dog serum. Lane B, infected dog serum. Lane C, immune rabbit serum. Lane D, normal rabbit serum. The numbers to the side are molecular weights.

Anti-erythrocyte membrane antibodies (IgG, IgM) were detected in sera of *B. gibsoni*-infected dogs by ELISA technique. Wells *et al.* [17] have reported a wide range of percentage of mononuclear cells destroyed by anti-lymphocytotoxic antibodies in sera of patients infected with either *P. falciparum* or *P. vivax*. In the present study, the individual sera of infected dogs exhibited a wider range of ELISA levels than those of normal dogs. Presumably, the wide range of ELISA levels may be due to the difference in stage of infection among the infected dogs. The mean ELISA level of anti-erythrocyte membrane IgG antibodies was higher than that of anti-erythrocyte membrane IgM antibodies. This result may support the previous study that the immunoglobulins associated with the erythrocyte membranes during *B. rodhaini* infection was mainly IgG [11]. There is another possibility that cold-reactive IgM antibodies might have been eluted from the erythrocyte membrane antigens during ELISA run at 37°C. In Western immunoblotting analyses, the presence of anti-erythrocyte membrane antibodies (IgG) was confirmed by the similarity in the protein profiles of the erythrocyte membrane antigens immunoblotted with rabbit anti-

serum to dog erythrocyte membrane antigens and infected dog serum. Furthermore, anti-erythrocyte membrane antibodies in IDS were found to be predominantly directed against a component with molecular weight of 48,000 daltons, and to react with even the components unrecognized by any antibodies in IRS. These results strongly indicate the production of autoantibodies to intact erythrocyte membranes in *B. gibsoni* infection.

Regarding the mechanism of anemia during *B. gibsoni* infection, besides mechanical destruction, anemia caused by a cellular immunologic mechanism has also been reported by Murase and Maede [12]. However, the degree to which a humoral immunologic mechanism contributes to anemia is unknown.

The present study has demonstrated the presence of anti-erythrocyte membrane antibodies in sera of dogs infected with *B. gibsoni*. This result is consistent with the previous reports that a positive coombs' test result was frequently encountered in dogs infected with *B . gibsoni* [3–5, 8]. Additional studies on *B. gibsoni* infection will be necessary to determine the *in vivo* relationship between anti-erythrocyte membrane antibodies and the destruction of erythrocytes.

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