Changes in Anti-Erythrocyte Membrane Antibody Level of Dogs Experimentally Infected with Babesia gibsoni

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ABSTRACT. To account for the conflict between the excessive destruction of erythrocytes and the number of parasitized erythrocytes in dogs infected with Babesia gibsoni, we examined the correlation between anti-erythrocyte membrane antibody level (AEMAL) and the number of erythrocytes (RBC count) in dogs with experimentally induced babesiosis using hematological examination and an enzyme linked immunosorbent assay (ELISA). In the infected dogs without splenectomy, more prominent reduction in RBC count accompanied with the elevated AEMAL was presented than anticipated from parasitemia until the 21st day. Furthermore, autoagglutinated erythrocytes and spherocytes were demonstrated in blood films. These results suggest that a humoral immunologic mechanism may be involved in a decrease in RBC count in dogs infected with B. gibsoni. —KEY WORDS: antibody to erythrocyte membrane, Babesia gibsoni infection, dog.

Babesia gibsoni, a causative agent of canine babesiosis, is a tick-borne hemoparasitic protozoan in the intrarhythrocytic stages or piromasts of it account for peracute, acute, or chronic anemia as the primary clinical findings. The mechanism for acceleration of erythrocyte destruction has not yet been elucidated in detail. Our clinical experience has revealed that erythrocyte destruction occurs in excess of that anticipated from the number of parasitized erythrocytes in the peripheral blood. Anemia uncorrelated with parasitemia has been seen in Plasmodium berghei [2], B. rohdei [14], and Anaplasma marginale [10] infections. Kanbara et al. [9] suggested that the elevated frequency of erythrocyte destruction in the course of P. berghei and B. rohdei infections were attributed in part to phagocytosis of erythrocytes opsonized with anti-erythrocyte antibodies by the mononuclear phagocytes. In B. gibsoni infection as well, the possibility for anti-erythrocyte antibody-mediated acceleration of erythrocyte destruction is worth taking into consideration in that a positive Coombs’ test result [4-6, 8] and spherocytes [5, 6] are sometimes encountered in dogs infected with B. gibsoni. Regarding the mechanism for anemia, the excessive hemolysis has been explained on the basis of a direct toxic effect of hemolytic factor(s) on the erythrocytes by Onishi et al. [12]. Murase and Maede [11] have reported on the basis of a cellular immunologic mechanism that erythrocytophagocytic activity of macrophages might be increased in B. gibsoni infection, however, it has not yet been substantiated that anti-erythrocyte antibodies participate in an immune mechanism for excessive erythrocyte destruction during low parasitemia. To elucidate the mechanism for anemia in B. gibsoni infection, our present study was designed to examine the correlation between AEMAL and RBC count following experimental infection.

B. gibsoni utilized in this study was originally isolated from a dog (D 1) diagnosed as babesiosis at the Veterinary Teaching Hospital, Miyazaki University in Japan. Parasites were maintained in our laboratory by passage in a dog (D 2). Both D 1 and D 2 were free of microfilaria and Rickettsia. The male mongrel dogs used in the present study, 2 years of age, weighing ca. 10 kg harbored no B. gibsoni, microfilaria and Rickettsia. One splenectomized dog (dog C) and two non-splenectomized dogs (dog D and E) were injected intravenously (i.v.) with approximately 7×10⁶ erythrocytes with a parasitemia of 40% from D 2. Two dogs (dog A and B) as controls were inoculated i.v. with approximately 7×10⁶ normal erythrocytes from D 2 before passage in D 2. Dog E was given an antibabesial drug, diminaeaz diacetate on the 21st day. Each dog had blood drawn at some intervals. RBC count and MCV were determined by the electronic method. The number of parasitized erythrocytes was counted in 1,000 erythrocytes in a Wright’s-Giemsa stained peripheral blood film and parasitemia was calculated. Hematological examination was carried out on the same smear that was used for calculation of parasitemia. Sera obtained from subjects were stored at −20°C till ELISA. Following dextran sedimentation [1], blood from a non-infected dog was applied to CF-cellulose (sigma) column to remove leukocytes. Ghost membrane was prepared according to the method of Tomoda, et al. [15]. The protein concentration of ghost membrane was assayed with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, California). Preparation was stored at −70°C until use. ELISA was performed by a modification of the method of Ishikawa et al. [7]. The microplate (Falcon 3912, Becton Dickinson, CA. U.S.A.) was covered with 0.05 ml of erythrocyte antigens (20 μg protein/ml) diluted in 0.05 M carbonate buffer (pH 9.6), and incubated at 4°C for 12 hr. After the washing of the plate with PBS, 0.1 ml of PBS containing 0.05% Tween 20 and 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 ml 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 ml of peroxidase-conjugated rabbit anti-dog immunoglobulins (IgG) (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. 0.1 ml of o-phenylenediamine dihydrochloride (Nacalai Tesque, INC., Kyoto, Japan) at a concentration of 0.4 mg/ml 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 ml 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).

As shown in Fig. 1, normal controls exhibited normal RBC counts during the experiment. In dog A, moderate increases in AEMAL were observed on the 4th, 7th and 21st day after inoculation with normal erythrocytes. In dog B as well, a slight increase in AEMAL was recognized on the 21st day. Nevertheless, the variation in AEMAL, presumably ascribed to the difference in blood type among individual dogs, was not associated with time course of RBC count within the normal range. In dog C, AEMAL, reaching a peak value on the 10th day, dropped until death. There was no significant difference in a peak value of AEMAL between dog A and C, for which splenectomy may account. AEMAL was not correlated with RBC count, while a prominent increase in parasitemia after the 14th day was associated with a significant decrease in RBC count. This indicates that drastic reduction in RBC count after the 14th day may be attributed to extensive mechanical destruction by escaping parasites. In dog D, RBC count below the normal range was observed on the 10th day. It reached a minimum on the 21st day when dog D died. AEMAL had prominently risen until the 21st day. An increase in parasitemia was observed on the 14th day. It reached 12% on the 21st day. Parasite-infected spherocytes were found on the 14th day. (Fig. 2. In dog E, RBC count and AEMAL showed the same trend as in dog D until the 21st day when dog E was given an antibabesial drug, diminazene diaceturate. Thereafter, RBC count increased along with reduction in AEMAL. Except on the 14th and 21st day when parasitemias reached 3%, parasitemia had been extremely low all through the experiment. Spherocytes and autoagglutinated erythrocytes were recognized on the 21st and 28th day (Fig. 2). On the 28th and 35th day, even polychromatophilic rubricytes showed up in the peripheral blood, reflecting strong bone marrow response to anemia (Fig. 2). In both dog D and E, a significant decrease in RBC count on the 10th day was correlated with a notable increase in AEMAL, however, no correlation was observed between RBC count and parasitemia. Although an increase in parasitemia was observed in dog D after the 10th day, parasitemia in dog E was still extremely low. That may be why anemia in dog D advanced more prominently than in dog E. A moderate recovery from anemia in dog E after the 21st day was correlated with reduction in AEMAL. These results suggest that the elevated AEMAL as well as high parasitemia may be involved in reduction in RBC count.

Partial phagocytosis of erythrocytes coated with autoantibodies by macrophages results in the formation of spherocytes [8]. Spherocytes may also be deparasitized erythrocytes with parasites pitted in spleen [3, 13].
However, *B. gibsoni* is difficult to pit from erythrocytes because of its small size. [5]. Spherocytes harboring parasites were found in our present study, indicating that spherocytes may have been formed through partial phagocytosis of erythrocytes coated with autoantibodies. Spherocytes are destroyed before long. Hence, the presence of spherocytes in the blood films of non-splenectomized dogs suggests the autoantibody-mediated anemia. The absence of spherocytes was observed in dog C throughout the experimental period, indicating that AEMAL was insufficient for the formation of spherocytes. There is an alternative possibility that antibodies-coated erythrocytes were free from phagocytosis because of splenectomy.

Granting that strong bone marrow response to anemia and a drop in AEMAL were observed in dog E after the 21st day, the degree of recovery from anemia was a little greater than was expected from AEMAL. Murase and Maede [11] reported that erythrophagocytic activity of macrophages is decreased by diminazene diaceturate, indicating that erythrocytes coated with antibodies may have passed through the spleen without undergoing a complete or partial phagocytosis by macrophages.

In the present study, it was found that RBC count, not necessarily associated with parasitemia, was inversely correlated with AEMAL. We could not confirm that anti-erythrocyte antibodies have opsonic activity in erythrophagocytosis, however, demonstration of parasite-infected spherocytes and autoagglutinated erythrocytes suggests that autoimmunity may be involved in anemia. Additional studies on *B. gibsoni* infection will be necessary to determine the relationship between an immunologic mechanism and erythrocyte destruction in detail.

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REFERENCES