Serum Hemolytic Activity of *Babesia gibsoni*-Infected Dogs: The Difference in the Activity between Self and Nonself Red Blood Cells

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ABSTRACT. The serum hemolytic activity of *Babesia gibsoni*-infected dogs varied when assayed with nonself red blood cells from different dogs, whereas it did not vary when assayed with red blood cells, irrespective of self or nonself, from a particular dog throughout the experiment. The variety in activity determined with nonself red blood cells was not related to the type of red blood cell by DEA, D and J systems. Serum hemolytic activity with self red blood cells was different in the course of infection from that with nonself red blood cells, especially in the late stage of infection, when the activity with self red blood cells decreased more rapidly than that with nonself red blood cells. The results indicate that the serum hemolytic activity of *B. gibsoni*-infected dogs determined with self red blood cells probably reflects the *in vivo* activity, suggesting that the rapid decrease in activity in the late stage of infection is a way of acquired resistances for the host to recover from hemolytic anemia in the infection. The facts that the hemolytic activity increased by heating the serum at 56°C, that the osmotic fragility of red blood cells remained almost on the same during the course of infection and that Coobms' test for red blood cells of the infected animal was negative suggest that the immune-mediated hemolytic anemia is not a possible mechanism for the progressive and severe anemia in *B. gibsoni*-infection. The present results support the previous notion that the increased serum hemolytic activity is at least one of the causes of anemia in canine *B. gibsoni*-infection.—KEY WORDS: anemia, *Babesia gibsoni*, blood cell type, osmotic fragility, serum hemolytic activity.

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Hemolytic anemia is a main clinical sign in canine *Babesia gibsoni* infection. Although several possible causes have been suggested for anemia in the infection, the pathogenesis of the progressive and severe anemia in the infection has not yet been clearly understood. Previously we reported that hemolytic activity in serum significantly increased after dogs were infected with *B. gibsoni* [7]. The activity is well correlated with parasitemia and anemia in both naturally and experimentally infected dogs. These findings indicate that serum hemolytic factor(s) possibly plays an important role in the development of the anemia in canine *B. gibsoni* infection. In the previous study, however, the serum hemolytic activity in *B. gibsoni*-infected dogs was assayed with red blood cells obtained not from infected dogs but from uninfected ones. This raises a question whether the serum hemolytic activity determined *in vitro* with nonself red blood cells reflects that in *in vivo* or not. In the present study, therefore, we examined the difference between the activities assayed with self and nonself red blood cells. The results show that the serum hemolytic activity in *B. gibsoni*-infected dogs assayed with nonself red blood cells varied depending on the donors of cells, whereas the activity assayed with red blood cells, irrespective of self or nonself, from a particular dog did not differ throughout the course of experiment.

Furthermore, in the present study, we examined the heating effect of serum on hemolytic activity and the change in osmotic fragility of red blood cells during the infection, and also applied Coombs' test to detect autoantibody on red blood cells to see whether autoimmune hemolysis participates in the occurrence of hemolytic anemia in *B. gibsoni* infection.

MATERIALS AND METHODS

Animals: Dogs used were obtained from the same sources and maintained as described previously [7]. Dogs were experimentally infected with *B. gibsoni* by intravenous transfusion of heparinized blood containing 4 to 5×10^7 infected cells per m*l* at a dose of 1 m*l*/kg of body weight as described previously [7]. As controls, heparinized blood taken from a clinically healthy dog was transfused to another healthy ones at a dose of about 0.2 m*l*/kg of body weight so as to transfer the same number of red blood cells as used to induce experimental infection.

Preparations of red blood cells and serum: To prepare red blood cells used for the assay of hemolytic activity, blood was taken from the external juglar vein or from the cephalic vein into a syringe containing 1/10 vol of 1% disodium ethylenediaminetetraacetic acid and was centrifuged at 550 × g for 10 min, and buffy coat was removed. Packed red cells were washed three times with phosphate-buffered saline (PBS, 10 mM NaH_2PO_4-Na_2HPO_4−155 mM NaCl, pH 7.4) containing 0.1% bovine serum albumin (PBS-BSA) and finally once again with PBS-BSA. The red blood cell preparations were kept at 4°C as a pellet and used within 24 hr. After removing red blood cells, the supernatant was heated at 56°C for 30 min and centrifuged at 1,200 × g for 10 min. To examine the effect of heating, a part of the serum was centrifuged without heating. The serum samples were stored at 4°C and used

for assay.

Assay of serum hemolytic activity: The condition for the assay of hemolytic activity was essentially the same as described previously [7]; the reaction mixture contained 0.3 m*l* of pelleted red blood cells, 0.5 m*l* of serum and 0.2 m*l* of PBS-BSA. After incubated at 37°C for 90 min, the mixture was centrifuged at 1,200 × g for 10 min at 4°C and the absorbance of the supernatant was determined at 540 nm. The supernatant of the mixture containing the same ingredients was used as a blank. Hemolytic activity unit was defined arbitrarily as an increase of 0.001 in absorbance.

Blood cell types: Blood types were identified by DEA, D and J systems [1, 3, 8, 9].

Assay by Coombs' test: Direct Coombs' test for the assay of red blood cells was carried out at 37°C with a mixture of rabbit anti-canine IgG, IgM and C_3 sera, which were purchased from Vetchek, INC Immuno-Biologicals, CA., U.S.A.

Assasy of osmotic fragility: The osmotic fragility of red blood cells was determined by the multiple tube method [4]. The percentage hemolysis in each tube was plotted against the percentage of sodium chloride concentration.

Other assay methods: Anemia was evaluated with the percentage decrease in hematocrit value. Parasitemia was assayed by counting the parasites in 1,000 red blood cells in Giemsa-stained thin blood smears.

Statistical analysis: Statistical significance of correlation coefficient was determined by F-test.

RESULTS

In our preliminary experiment, serum hemolytic activity occasionally varied between *B. gibsoni*-infected dogs, when assayed with red blood cells from several different dogs. Therefore, in the previous study, we used only two dogs as the donor of red blood cells. Serum hemolytic activities of three infected dogs were determined with red blood cells from 10 uninfected and 3 infected dogs, from which sera were prepared. The activity of each serum assayed with nonself red blood cells varied largely between different donor dogs of red blood cells (Table 1). In contrast, when determined with the red blood cells prepared from the same animal, irrespective of self or nonself blood cells, the serum hemolytic activities of infected and uninfected animals were confined within a relatively narrow range (Table 2). The variety in serum hemolytic activity assayed with nonself red blood cells of different animals was not correlated with the types of red blood cell (Table 1).

The variety in serum hemolytic activity depending on donor dogs of red blood cells suggested that the activity assayed with nonself red blood cells, even when prepared from a particular dog, did not reflect the *in vivo* activity. Therefore, we examined the change in serum hemolytic activity assayed with self or nonself red blood cells in experimental *B. gibsoni* infection. Figure 1-a shows the activities assayed with self and nonself red blood cells for

Table 1. Serum hemolytic activity of *B. gibsoni*-infected dogs assayed with nonself red blood cells from different dogs and their red blood cell types

Dog No[a]	Hemolytic activity[b] in serum of dog.			Blood type											
				DEA system						D system		J system			
	No. 5	No. 8	No. 11	1 1	1 2	3	5	6	D1	2	J1	2	3	4	5
1	87	34	51	+[e]	−	−	−	−	−	+	−	+	−	−	−
2	66	52	20	+	−	−	−	−	−	+	−	+	−	−	−
3	62	68	18	+	−	+	−	−	−	+	−	+	−	−	−
4	53	37	20	+	−	−	−	−	−	+	−	+	−	−	−
5	−[c]	34	35	+	−	−	−	−	−	+	−	+	−	−	−
6	51	35	30	+	−	−	−	−	−	+	−	+	−	−	−
7	39	38	15	+	−	−	−	−	−	+	−	+	+	−	−
8	31	−[c]	69	+	−	−	−	−	−	+	−	−	−	−	−
9	29	8	31	+	+	−	−	−	+	+	−	+	−	−	−
10	25	45	50	+	−	−	−	−	−	+	−	+	−	−	−
11	20	35	−[c]	+	−	−	+	−	−	+	−	+	+	+	−
12	6	46	0	+	−	−	−	−	−	+	−	+	−	−	−
13	0	41	20	+	−	−	+	−	−	+	−	+	−	+	−
$\bar{X}$[d]	39 82	39 42	29 92												
SD	26 76	13 96	18 97												

a) Donor dogs of red blood cells for the determination of hemolytic activity and for the blood type.
b) Sera were prepared from *B. gibsoni*-infected dogs Nos. 5, 8 and 11, respectively.
c) The activity assayed with self red blood cells was omitted from the data.
d) Hemolytic activities (mean±standard deviation) determined with red blood cells of dog Nos. 1 to 13.
e) +, positive in blood cell typing; −, negative in the typing.

Table 2. Serum hemolytic activity of *B. gibsoni*-infected and -uninfected dogs determined with self and nonself red blood cells

Serum of;	Hemolytic activity with[c,d];		
Uninfected dog[a]	Self RBC	9.43± 0.90	(7)
	Nonself RBC	17.6 ± 5.92	(14)
Infected dog[b]	Self RBC	56.25± 5.07	(4)
	Nonself RBC	80.2 ±17.5	(4)

a) Serum was prepared from a particular dog, from which self red blood cells (RBC) were prepared.
b) Serum and self red blood cells were prepared from a particular dog on day 18 after the experimental infection with *B. gibsoni*.
c) Red blood cells (RBC) and sera were prepared on the same day to assay the activity. Nonself RBC was prepared from different uninfected animals.
d) Data represent mean±standard deviation. Figures in parentheses indicate the number of determinations.

34 days after infection. The time course of the changes in activity determined with nonself red blood cells was essentially the same as described in the previous report [7]. In contrast to this, although the activities determined with self or nonself red blood cells increased almost parallel to each other before reached the maximum, the activity with self red blood cells decreased more rapidly than that with nonself red blood cells in the late stage of the infection; the activity with nonself red blood cells

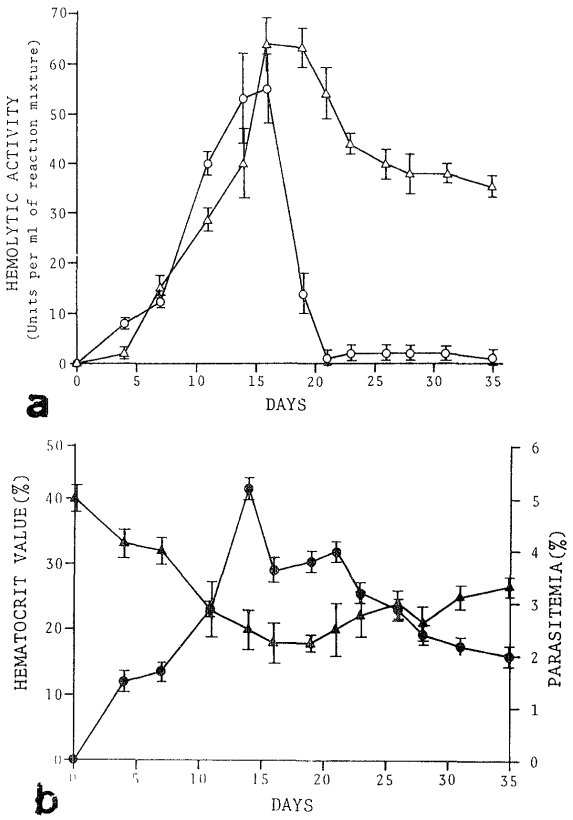

Fig. 1. Serum hemolytic activity of *B. gibsoni*-infected dogs was determined with self and nonself red blood cells. (a) Dogs were experimentally infected with *B. gibsoni* and the serum and the red blood cells were obtained from a particular dog on the same day to determine the activity with self red blood cells. The hemolytic activity of the same serum was also determined with nonself red blood cells, which were obtained from a particular uninfected dog, throughout the experiment. Prior to the experiment, an uninfected dog was selected to give relatively high activity to the serum of infected animal. (b) Hematocrit value and parasitemia were also determined with the blood obtained from the same infected animal for the determination of hemolytic activity. Vertical bars represent the standard error of means of 4 animals. Symbols: ○, serum hemolytic activity assayed with self red blood cells; △, the activity with nonself red blood cells; ▲, hematocrit value and ●, parasitemia.

reached the maximum slightly later than that with self red blood cells and then gradually decreased. The serum hemolytic activity determined with nonself red blood cells correlated well to the number of parasites in blood and the hematocrit value during the infection (Fig. 1-b). On the other hand, the activity determined with self red blood cells did not correlate to those indices of infection. The correlation coefficients between the activity assayed with nonself red blood cells and parasitemia and between the activity and hematocrit value were 0.81 and −0.93, respectively, which were statistically significant at a level of 1%. The correlation coefficients between the activity

Table 3. Effect of heating of serum on hemolytic activity in the serum of *B. gibsoni*-infected and -uninfected dogs

Serum of;	Serum hemolytic activity	
	Heated[c]	Not heated
Uninfected dog[a]	31.4±1.34 (5)	23.6±5.59 (5)
Infected dog[b]	53.5±4.43 (4)	38.8±2.99 (4)

a) Red blood cells and sera were prepared from a particular dog.
b) Red blood cells and sera were prepared from the dogs on day 15 after the experimental infection with *B. gibsoni*.
c) Sera were heated at 56°C for 30 min.
d) Data are represented by mean±standard deviation. Figures in parentheses indicate the number of determinations.

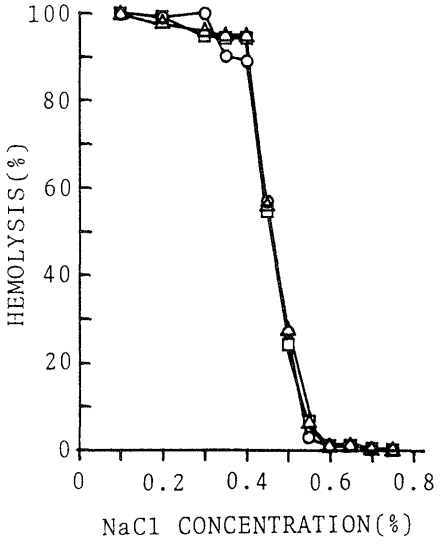

Fig. 2. Osmotic fragility curves of *B. gibsoni*-infected dogs. Red blood cells were obtained before (○) and on days 17 (△) and 24 (□) after infection.

with self red blood cells and parasitemia and between the activity and the hematocrit value were 0.55 and −0.42, respectively, which were not significant at a level of 5%. During the course of infection, hemolytic activity determined with red blood cells of infected dogs and the serum from an uninfected dog remained almost constant in level; the serum activities before and 16 and 21 days after infection were 6.7 ± 2.2, 5.0 ± 1.8 and 7.0 ± 2.8 (mean ± standard deviation, n=4), respectively.

To see whether complements play a role in the increase in serum hemolytic activity of *B. gibsoni*-infected dog, the sera prepared from *B. gibsoni*-infected and -uninfected dogs were heated at 56°C for 30 min and the activities were determined with self red blood cells from these dogs (Table 3). The activity of sera from both animals increased but did not decrease by heating the sera.

To examine whether hemolytic anemia is caused by the instability of red blood cells in *B. gibsoni*-infection, the osmotic fragility of the cells was determined. The osmotic

fragility curves of red blood cells were almost the same between before and on days 17 and 24 after infection (Fig. 2). All red blood cells obtained from these animals were negative in the assay for the direct Coombs' test.

DISCUSSION

The present results show that the serum hemolytic activity of *B. gibsoni*-infected dogs varies depending on the donor dogs of red blood cells. However, the activity was reproducible when the cells, irrespective of self or nonself, were prepared from a particular dog throughout the course of experiment, although hemolytic activity assayed with nonself red blood cells varied between different donor dogs. At present, it is not clear why serum hemolytic activity of infected dogs varies between the red blood cells from different dogs. As shown in the present experiment, the difference is not due to the type of red blood cell. Hemolytic activity seems to vary according to the difference in affinity for binding site of the serum hemolytic factor(s) on the cell membrane depending on the source of the cells, because hemolysis is probably initiated by the binding of the factor(s) on the membrane of red blood cells.

In *B. gibsoni*-infection, the hemolytic activities assayed with nonself red blood cells obtained from a particular uninfected dog throughout the experimental period were inversely proportional to hematocrit values, whereas those with self red blood cells from infected dogs increased inversely proportional to the values until the activity reached the maximum, but thereafter they decreased rapidly. These results suggest that the activity determined with nonself or self red blood cells reflects the *in vivo* change in a different way. The serum hemolytic activity determined with nonself red blood cells from uninfected dogs seems to be correlated with the amount of hemolytic factor(s) in blood of infected dogs, because the activity is assayed with red blood cells of almost the same composition throughout the experiment. On the other hand, the activity assayed with self red blood cells from infected animals probably reflects actual *in vivo* changes in hemolysis, because both serum and red blood cells used for the assay are originated from the same animal and also the ratio between the volumes of serum and red blood cells is very similar to circulating blood in composition as described previously [7].

The time course of serum hemolytic activity was markedly different between self and nonself red blood cells in the late stage of *B. gibsoni*-infection; the activity assayed with the self red blood cells from infected dog decreased more rapidly than that with nonself red blood cells from uninfected dogs. Although the reason for the difference is not yet clear, the decrease in activity determined with self red blood cells is possibly due to the appearance of cells resistant to serum hemolytic factor(s) in circulating blood, because the same serum gave higher activity when assayed with nonself red blood cells from uninfected dogs. The decrease in activity in the infection possibly provides a period for the host to recover from continuous damages by progressive and severe hemolytic anemia. This is explainable as one of acquired resistances of host in *B. gibsoni*-infection, if infected dogs produce red blood cells resistant to hemolytic factor(s).

It has been reported that hemolytic anemia in *B. gibsoni*-infection is partly due to autoimmune or immune-mediated hemolysis [2]. However, in the present study, serum hemolytic activity of the infected dogs did not decrease but increased by heating serum before assay, this indicating that complement-mediated hemolysis is not involved in the assay of hemolytic activity and that the hemolytic factor(s) in serum of *B. gibsoni*-infected dogs is rather heat resistant. The present study also showed that the osmotic fragility of red blood cells from *B. gibsoni*-infected dogs remained at the same level during the experimental period. In addition, Coombs' test was negative to red blood cells used for fragility test. All these results indicate that immune-mediated hemolysis is not a possible mechanism of anemia in *B. gibsoni*-infected dogs. Recently, Murase and Maede reported the increase in clearance of aged red blood cells by macrophages activated by *B. gibsoni* infection [5]. They also reported the multiplication of the parasite in *in vitro* culture, which is accompanied by the increase in hemolysis of nonparasitized red blood cells [6]. These findings show that hemolytic anemia in *B. gibsoni* infection is caused sequentially by invasion and multiplication of the protozoa in the red blood cell and then by the destruction of cells including nonparasitized cells. It seems likely that the hemolytic factor(s) described in the present report plays a role in the hemolysis of nonparasitized red blood cells.

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