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## MITOGENIC RESPONSIBILITIES OF LYMPHOCYTES IN CANINE BABESIOSIS AND THE EFFECTS OF SPLENECTOMY ON IT

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In order to clarify the mechanisms of immune-responses in canine babesiosis, mitogen-induced transformation of lymphocytes from peripheral blood and its relation to parasitemia were chiefly examined in both intact and splenectomized dogs suffering from *Babesia gibsoni*. The lymphocyte responses to PHA-P, Con A and PWM in dogs infected with *B. gibsoni* were all extremely depressed as the parasite increased in their peripheral blood. The lymphocyte responses to the mitogens greatly decreased after splenectomy in both groups infected and non-infected with *B. gibsoni*. Furthermore, severe relapse of parasitemia appeared in the infected dogs after splenectomy. Mild parasitemia and moderate anemia were prolonged for at least 3 months with a high serum IFA titer to the parasite in splenectomized dogs.

Key words : *Babesia gibsoni*, canine babesiosis, canine lymphocytes, splenectomy

### INTRODUCTION

It has been thought that cell-mediated immune response mainly acts to protect the host against the parasite,<sup>4,11)</sup> although antibody-mediated response has also been suggested to be important to ensure the elimination of the parasite and the action of cell-mediated immunity.<sup>12)</sup> Furthermore, it is known that the spleen plays a major role in inhibiting the proliferation of the parasite in the host's organs.<sup>1,9,12)</sup> However, the immune responses and the role of the spleen of the host animals to *Babesia* infection are still obscure. Thus in the present study, we investigated the cellular immunologic status of dogs infected with *Babesia gibsoni* by evaluating the responses of peripheral blood lymphocytes to nonspecific mitogenic stimuli, and also examined the effect of splenectomy on lymphocyte function.

### MATERIALS AND METHODS

#### *Experimental animals and Protozoa*

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Four healthy dogs, 1 to 3 years, were used. All of the dogs were born and kept in an area in Hokkaido where *Babesia gibsoni* infection in dogs had not been recognized. Two of the dogs were splenectomized at 30 days before the inoculation of *B. gibsoni*. Splenectomy was also carried out in the remaining 2 dogs, which recovered from experimental acute babesiosis at about 1 year after the inoculation. The strain of *B. gibsoni* used in this experiment was obtained originally from a dog infected naturally with *B. gibsoni* in Nagasaki City in 1973 and was maintained in dogs at Hokkaido University. Approximately 5ml of blood from a carrier dog were injected intravenously into each of the experimental dogs.

#### *Preparation of canine lymphocytes*

Approximately 3ml of peripheral blood from each dog were collected using a plastic syringe containing heparin (20 unit per ml of blood) and diluted 1:3 with a phosphate-buffered saline (PBS; pH. 7.2). About 8ml of the diluted blood sample were layered on a Ficoll-Conray solution with a density of 1.082, which was prepared by mixing 9 or 12% Ficoll solution (Ficoll 400, Pharmacia, Sweden) with 27.9 or 33.4% Conray solution (Conray 400, Daiichi Chem. Co., Japan), and centrifuged for 60min at 700g at 20°C. After the centrifugation, the mononuclear cells were removed and washed with PBS two times, and the cell concentration was adjusted to  $5 \times 10^5$  cells per ml.

#### *Lymphocyte blastogenesis test*

In the test, 0.2ml of the cell suspension was added to flat bottom tissue culture microtiter plates (Corning) containing RPMI 1640 medium (Gibco), 10% inactivated fetal bovine serum (FBS) and antibiotics (100 units penicillin G and 100  $\mu$ g streptomycin per ml) with optimum concentrations of PHA (phytohemagglutinin-P, Difco), Con A (concanavalin A, Difco) and PWM (pokeweed mitogen, Gibco). In our preliminary experiments, it was determined that 1  $\mu$ l of PHA, 5  $\mu$ g of Con A and 10  $\mu$ l of PWM per ml gave optimal lymphoproliferative responses with canine lymphocytes, respectively. The control cultures contained RPMI 1640 media with FBS and antibiotics but no mitogens. Microtiter plates with triplicate samples of lymphocytes for each mitogen were incubated in 5% CO<sub>2</sub> in air at 37°C for 72 hours in a humidified incubator. After 48 hours' incubation, 0.5  $\mu$ Ci of tritiated thymidine was added to each well containing cells and incubated for an additional 24 hours. After the incubation, samples were harvested with an automatic cell harvester (Labo Mash, Lab. Science, U.S.A.), and filter pads containing labeled cells were dried and placed in Aquasol (NEN, Boston, U.S.A.) and counted in a liquid scintillation spectrophotometer (Aloka LSC651, Aloka, Japan).

Data were expressed as mean counts per minute (cpm) and/or mean stimulation indices (SI), which were obtained by dividing the mean cpm of triplicate culture with mitogen by the mean cpm of triplicate cultures without mitogen (control).

#### *Antibody detection*

The serum antibody levels in infected dogs were detected by the indirect fluorescent antibody (IFA) method described by Leeftang and Perie<sup>8</sup> with slight modifications. In this test, FITC-conjugated rabbit IgG directed against dog IgG (Myles-Yada Ltd /, Israel) was used.

## RESULTS

1 *Lymphocyte blastogenesis test in normal dogs*

In the preliminary experiment, we carried out lymphocyte blastogenesis tests 5 times in 4 normal dogs at 7-day intervals. The results revealed that lymphocyte responses to nonspecific mitogens (PHA-P, Con A, PWM) varied with the individual but that the responses were almost stable in each dog throughout the experiment (Data not shown). The results indicated that the test can be employed as an in vitro assessment of lymphocyte function in dogs. Four dogs (Nos. 1-4) were then used for the following experiments.

2 *Immunoresponsiveness in dogs with babesiosis*

1) Lymphocyte responses to the mitogens. After the inoculation of *B. gibsoni*, the dogs (No. 1 & 2) showed a gradual decrease in [<sup>3</sup>H] thymidine uptake and / or SI. The decrease of the lymphocyte responses to PHA and Con A were more marked than the response to PWM. At 16-22 days after the inoculation, both [<sup>3</sup>H]-thymidine

TABLE 1 The changes of the lymphocyte responses to mitogens in a dog (No. 1) infected with *Babesia gibsoni*

Days*	Without Mitogen	Mitogens		
		PHA-P	CON A	PWM
-10	209 ± 56**	22,461 ± 2,852	22,173 ± 1,753	8,102 ± 513
- 2	193 ± 94	25,007 ± 1,422	23,148 ± 2,602	7,947 ± 346
<i>B. GIBSONI</i> inoculation				
1	193 ± 29	19,693 ± 2,286	19,801 ± 1,656	6,707 ± 222
4	190 ± 104	18,373 ± 1,230	21,421 ± 1,655	5,706 ± 598
7	214 ± 103	16,923 ± 754	20,167 ± 1,245	4,673 ± 121
10	230 ± 61	15,557 ± 1,134	14,339 ± 713	3,842 ± 230
14	190 ± 65	5,934 ± 1,030	11,962 ± 462	ND
18	441 ± 62	21,779 ± 1,655	21,138 ± 1,023	9,178 ± 231
22	375 ± 23	8,568 ± 1,222	10,809 ± 484	4,252 ± 309
26	271 ± 43	13,288 ± 1,215	11,043 ± 1,067	3,817 ± 1,685
40	200 ± 47	20,869 ± 2,044	21,372 ± 837	10,697 ± 808

\*Days after *B. gibsoni* inoculation, \*\*cpm: mean ± S.D., ND: Not done

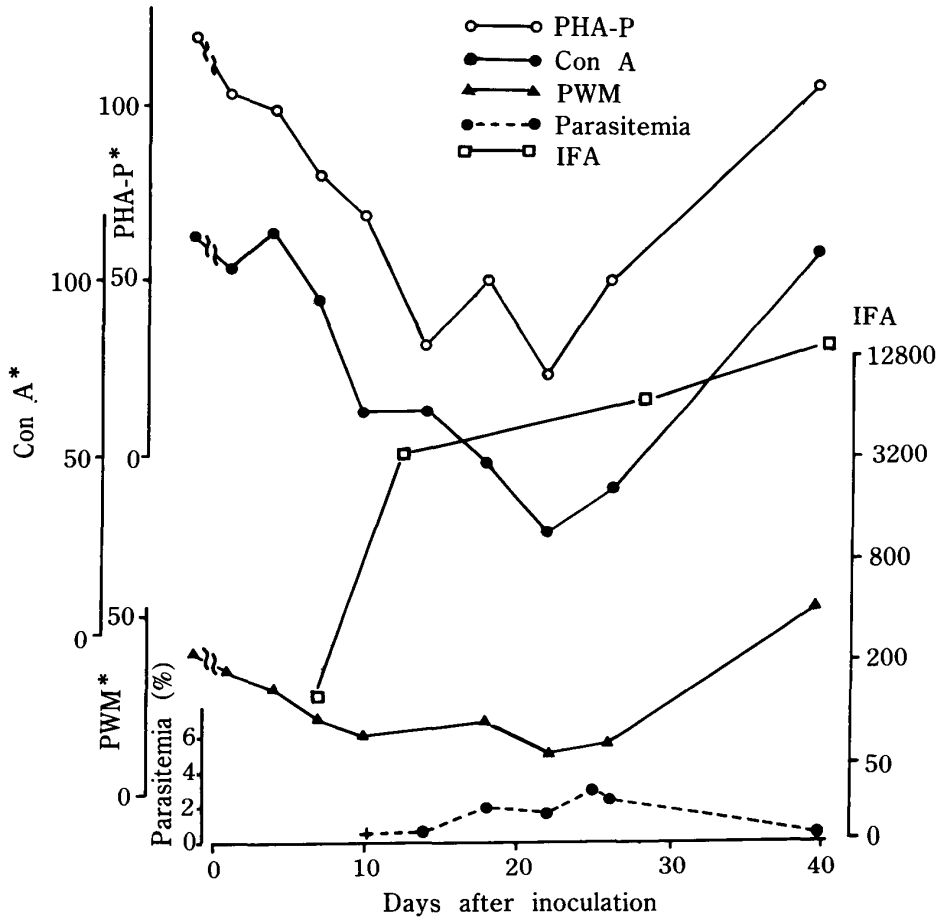


FIG. 1 Changes of lymphocyte responses to mitogens, parasitemia and indirect fluorescent antibody (IFA) in a dog (No. 1) infected with *Babesia gibsoni*.  
\*Data are expressed as mean stimulation indices (SI).

uptake and SI to each mitogen decreased to the lowest levels during the experimental period. Thereafter, they began to increase and reached their normal levels at 40 days. (Tab. 1 & Fig. 1).

2) Parasitemia and immunofluorescent antibody (IFA). *B. gibsoni* appeared in the peripheral blood of the infected dogs at 7 to 10 days after inoculation, and then gradually increased in number and reached a peak at 22–25 days. However, in two dogs (Nos. 1 & 2) parasitemia never exceeded 9.5%, and it almost disappeared by 40 days (Fig. 1).

The serum IFA titer rose rapidly after inoculation and reached a high level of 1:6400–1:12800 at 40 days (Fig. 1).

### 3 Effects of splenectomy on *Babesia* infection in dogs

1) Lymphocyte responses to the mitogens Two dogs (Nos. 3 and 4) were splenectomized before inoculation. Lymphocyte responses to each mitogen were markedly reduced after the splenectomy (Tab. 2) and did not return to their normal level for at least 4 weeks. Thirty days later, the dogs were inoculated with *B. gibsoni*. After that, [<sup>3</sup>H]-thymidine uptake and SI to each mitogen were still maintained at a low level in each dog (Tab. 2).

2) Parasitemia and IFA In these dogs, the parasites appeared in the blood at 2–4 days after the inoculation, and 10 days later, the dogs showed a high parasitemia (16–20%), which continued until the dogs died at 23 and 29 days, respectively.

The IFA titer did not exceed a level of 1:800 in each dog during the experimental period (Fig. 2).

#### 4 Effects of splenectomy on dogs that recovered from acute babesiosis

1) Lymphocyte responses to mitogens Two dogs (Nos. 1 & 2) were splenectomized at about 1 year after recovery from acute babesiosis. Before the splenectomy, lymphocytes from the dogs responded well to each mitogen and no parasites were observed in the blood. After the splenectomy, however, individual responses to the

TABLE 2 The effects of splenectomy on the lymphocyte responses to mitogens in a dog (No. 3) infected with *Babesia gibsoni*

Days*	Without Mitogen	Mitogens		
		PHA-P	CON-A	PWM
-44	162 ± 9**	4,039 ± 276	3,760 ± 379	1,203 ± 78
-40	181 ± 5	6,757 ± 270	7,644 ± 576	2,528 ± 279
splenectomy (-30)				
-28	115 ± 86	134 ± 49	145 ± 52	163 ± 60
-24	158 ± 67	810 ± 103	462 ± 15	681 ± 102
-20	142 ± 98	699 ± 177	700 ± 56	932 ± 179
-16	122 ± 33	551 ± 138	441 ± 39	440 ± 30
-12	112 ± 62	1,843 ± 212	1,670 ± 170	877 ± 77
-2	158 ± 78	557 ± 116	666 ± 63	476 ± 77
<i>B. gibsoni</i> inoculation				
4	180 ± 53	698 ± 129	831 ± 52	977 ± 179
8	201 ± 83	941 ± 99	634 ± 139	818 ± 271
12	417 ± 44	1,192 ± 361	1,187 ± 103	898 ± 160
16	316 ± 43	643 ± 30	581 ± 131	311 ± 153
19†	380 ± 84	819 ± 100	742 ± 107	644 ± 124

\*Days after *B. gibsoni* inoculation, \*\*cpm: mean ± S.D., †dies at 23th.

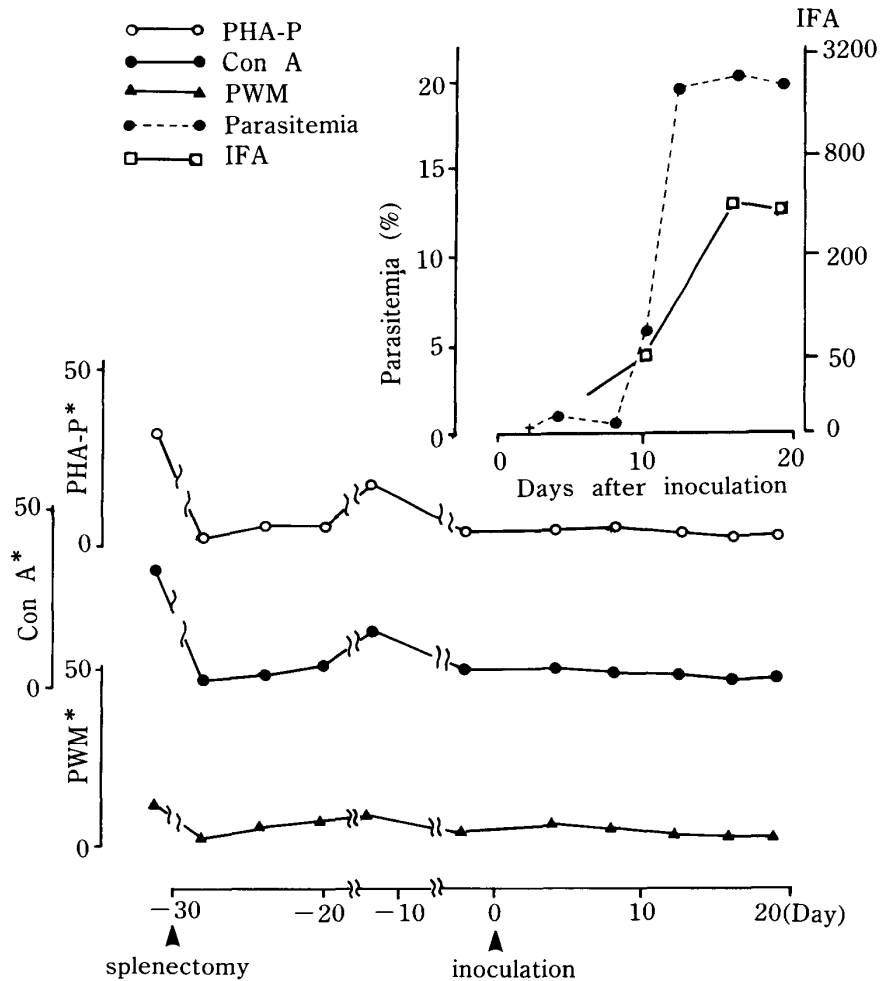


FIG. 2 Change of lymphocyte responses to mitogens, parasitemia and indirect fluorescent antibody (IFA) in a splenectomized dog (No. 3) infected with *B. gibsoni*.

\*Data are expressed as mean stimulation indices (SI).

mitogens were markedly reduced during 2–20 days, especially at 15 and 19 days in the two dogs, respectively. Thereafter, however, the responses to the mitogens began to increase and returned to the normal level at 30 and 40 days after the splenectomy (Fig. 3).

2) Parasitemia and IFA As the responses to the mitogens decreased; the parasites began to increase in the blood of each dog. In one dog (No. 1), the highest parasitemia (30.9%) was observed at 15 days and in another dog at 16 days (24.8%), corresponding well with the periods when each dog showed the lowest responses of lymphocytes to mitogens (Fig. 3). Parasitemia, thereafter, decreased in inverse

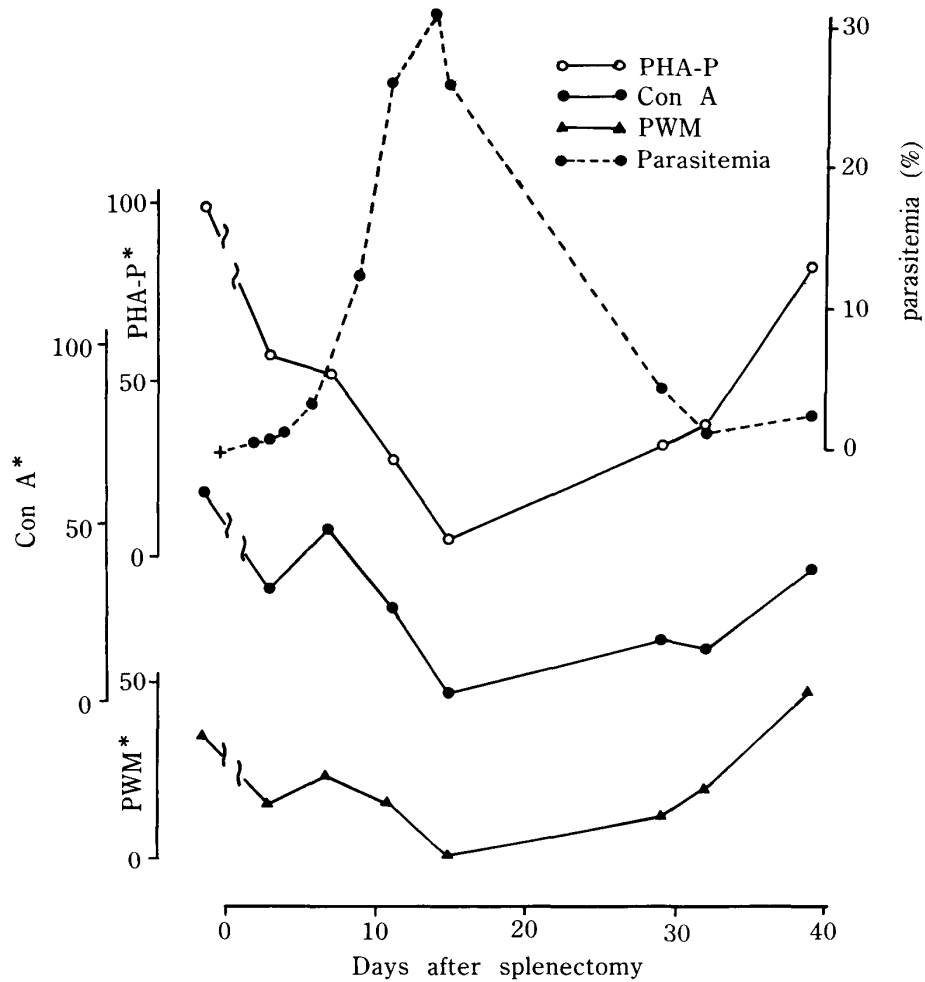


FIG. 3 Changes of lymphocyte responses to mitogens and parasitemia after splenectomy of a *B. gibsoni* carrier dog (No. 1). \*Data are expressed as SI.

proportion to the increase of the lymphocytes responses to mitogens in each dog (Fig. 3). In these dogs, anemia induced by the parasite was prolonged for at least 3 months. In addition, low parasitemia (0.4–1.8%) was also maintained for that period, despite the fact that a high level of IFA titer (1:12800 or more) was also maintained in each dog (Tab. 3).

TABLE 3 Lymphocyte responses to mitogens, parasitemia and IFA titer in 2 splenectomized dogs recovered from recrudescence of clinical babesiosis

Dog No.	Days	Parasitemia (%)	Hematocrit (%)	Lymphocyte Responses			
				PHA-P (SI)	Con A	PWM	IFA
No. 1	129*	0.9	13.0	65.8	34.5	29.1	12,800
No. 2	178*	1.8	15.0	25.7	13.4	14.5	12,800

\*Days after recrudescence of babesiosis

#### DISCUSSION

The nonspecific mitogens chosen for the present study, PHA-P, Con A and PWM, are commonly used in lymphocyte blastogenesis test, including these for the dog.<sup>3,7)</sup> Of the mitogens, PHA and Con A are generally cited as being T-lymphocyte specific. Krakowka and Ringler considered Con A to be a restricted T-cell mitogen in the dog on the basis of its inability to induce any B-cell response and of its ability to stimulate lymphoblasts possessing a specific T-cell antigen.

In the present study, lymphocyte responses to each mitogen, especially to Con A and PHA, were markedly suppressed during acute babesiosis and/or during the relapse of parasitemia after splenectomy in the carrier dogs. These results indicate that the infection of *Babesia* parasites induces an immunosuppressive state in the infected dogs, which may be due to decreased T-lymphocyte functions. Similar observations were made in humans with acute babesiosis. ROWIN et al.<sup>10)</sup> reported that the responses of lymphocytes to PHA, Con A and PWM in patients with babesiosis were strongly depressed during the acute-stage of the disease, while the mitogenic responsibility returned to normal following recovery from the illness. BENACH et al.<sup>2)</sup> also reported that the number of T-lymphocytes and responses to nonspecific mitogens were decreased during acute babesiosis in humans. These observations are consistent with the results of the present study, though the exact mechanism whereby babesia parasites suppress the T-cell function is yet unknown. BENACH et al. also reported that levels of  $T_{\gamma}$ -cell were elevated in patients with babesiosis.<sup>2)</sup> The  $T_{\gamma}$ -cells, a subpopulation of T-lymphocytes that possesses Fc receptors for IgG, have been shown to have suppressor / cytotoxic functions.<sup>6)</sup> Thus, BENACH et al. supposed that the increase of  $T_{\gamma}$ -cells in the response to babesia infection seems to be associated with the depressed responses of peripheral lymphocytes to mitogens in vitro.

In the present study, it was also demonstrated that the lymphocyte responses to

mitogens were markedly depressed after splenectomy in healthy dogs, that the decreased responses did not return to normal and that the dogs died as a result of severe infection by the parasites. These results strongly suggest that the post-splenectomy state causes consistent abnormality in peripheral lymphocytes, especially T-cell functions. On the other hand, the mitogenic responses were also depressed after splenectomy in the dogs carrying the parasite, and the dogs showed recrudescence of clinical babesiosis, but the decreased lymphocyte function returned to normal during convalescence from the disease. The reasons for this discrepancy are unknown at present. In this connection, it is known that the spleen is important for the induction of immune reactions operating in the effector branch of the immune response, but that once the immune reactions develop, immunity is retained, even if the spleen is subsequently removed.<sup>5)</sup> It thus appears that the immune status of the carrier dogs against *Babesia* parasites might play a major role in not only inhibiting the proliferation of the parasites but also in maintaining lymphocyte functions, even after splenectomy. This possibility, however, remains to be proved.

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