Abstract. We examined lymphoproliferative response to phytohemagglutinin and anti-parasite antibody level in dogs naturally infected with Babesia gibsoni. The dogs with subclinical B. gibsoni infection exhibited suppressed lymphocyte blastogenesis. Prominent depression of lymphocyte blastogenesis and anti-parasite antibody production was observed in dogs suffering from relapses of clinical B. gibsoni infection. — Key words: B. gibsoni, canine, immunosuppression.

Babesia gibsoni, a causative agent of canine babesiosis, is a tick-transmitted hemoparasitic protozoa. Although effective in retarding the clinical advancement of B. gibsoni infection, most antibabesial drugs can not eliminate the parasites from all infected dogs [11], thereby resulting in persistent subclinical infection. The development of subclinical B. gibsoni infection might depend on a delicate balance between the pathogenicity of parasite and the immune response of host [10]. However the triggers of a relapse of clinical B. gibsoni infection remain to be resolved completely. The present study was designed to investigate immune response in dogs naturally infected with B. gibsoni.

Six B. gibsoni-infected and 5 healthy control dogs, 1 to 5 years of age, were used in the present study. They were all admitted to the Veterinary Teaching Hospital, Miyazaki University in Japan. Three dogs (cases 1 to 3) were subclinically infected with B. gibsoni and the other 3 dogs (cases 4 to 6) suffered from relapses of clinical B. gibsoni infection.

Lymphocytes were obtained from the peripheral blood of dogs using lymphoprep (Daichi Pure Chemicals Co., Ltd., Japan) and examined for proliferative response to phytohemagglutinin (PHA) (Difco Laboratories, U.S.A.) by a glucose consumption test [5]. Lymphocytes were suspended at a rate of 5 x 10⁶ cells/ml in RPMI-1640 (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, U.S.A.), penicillin (100 IU/ml) and streptomycin (100 IU/ml). Twenty µl of PHA solution at a concentration of 100 µg/ml and 200 µl of the lymphocyte suspension were poured into each of 96 wells of a sterile flat bottom microplate (Corning, U.S.A.). Control culture contained only the lymphocyte suspension. The plate was incubated at 37°C in 5% CO₂ for 4 days. The glucose concentration of supernatant of each well was measured by the glucose oxidase method (Wako Pure Chem., Tokyo, Japan). Stimulation ratio (SR), an indicator of lymphocyte proliferation, was calculated using the following formula: SR (%) = (control culture (mg/dl)-stimulated culture (mg/dl))/control culture (mg/dl) x 100

Lymphocyte stimulation test was performed twice.

Anti-parasite antibody level in sera of the dogs was evaluated by an enzyme linked immunosorbent assay (ELISA). The antigen was prepared by mixing 20% parasitized erythrocyte suspension with an equal volume of 0.5% saponin, and the mixture was then centrifuged at 27,000 G for 30 min. The resultant sediment was rinsed and centrifuged with PBS four times at 27,000 G for 30 min. Parasite suspension in PBS was sonicated and centrifuged at 11,000 G for 30 min. The resultant supernatant was used as B. gibsoni antigen. ELISA was carried out following the previously reported method [9]. In brief, a microplate (Falcon 3912, Becton Dickinson, CA, U.S.A.) was coated with B. gibsoni parasite antigen (130 µg protein/ml) diluted in carbonate buffer (pH 9.6) and incubated at 4°C for 12 hr. After the plate was rinsed, 2% bovine serum albumin was added to each well, and the plate was incubated at 37°C for 1 hr. After rinsing the plate, test serum was added, and incubated at 37°C for 30 min. After rinsing the plate, peroxidase-conjugated goat anti-dog IgG (Heavy & Light chain specific, Cappel, PA, U.S.A.) was added, and incubated at 37°C for 30 min. After the plate was rinsed, 5-aminosalicylate was added and incubated at room temperature for 30 min. The reaction was stopped by addition of 2 M sulfuric acid. The well contents were measured for absorbance at 450 nm by a micro-ELISA spectrophotometer (Easy Reader, EAR 400 FW, STL-Labinstruments, Austria).

Control animal group consisted of a poodle (male, 5 years of age), a Doberman (female, 1 year of age), a retriever (female, 1 year of age), a bullock (female, 2 years of age) and a mongrel (male, 2 years of age). As shown in Table 1, anti-parasite ELISA levels of the controls were under an upper negative limit of 0.115 [9], indicating that control dogs harbored no B. gibsoni parasite.

Case 1 was a 4-year-old male mongrel dog. On his arrival at the hospital, he exhibited weakness, pallor of mucous membranes and anorexia. Blood examination revealed that RBC count was 150 x 10⁹/µl and parasitemia level was 6%. The dog was treated with diminazene diaceturate (DD) and thereafter recovered from the clinical B. gibsoni infection. Immune response, RBC count and parasitemia level were examined on day 30 post DD administration. RBC count increased to 470 x 10⁹/µl. Parasitemia level was extremely low (<0.1%). Case 2 was a 2-year-old female pointer. At the first examination, weakness, pallor of mucous membranes and anorexia were manifested. RBC count was 300 x 10⁹/µl and parasitemia level was 1%. She was treated with DD and thereafter recovered from clinical B. gibsoni infection. Immune response, RBC count and parasitemia level were examined on day 30 post DD administration. RBC count
Table 1. Lymphocyte stimulation ratio (SR) values and anti-parasite ELISA levels of B. gibsoni-infected dogs

<table>
<thead>
<tr>
<th>Dogs</th>
<th>No.</th>
<th>SR value (×10^6 μl)</th>
<th>ELISA level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogs with subclinical</td>
<td>3</td>
<td>11.95 ± 2.78*</td>
<td>0.27 ± 0.15*</td>
</tr>
<tr>
<td>infection (cases 1 to 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs suffering from</td>
<td>3</td>
<td>2.87 ± 1.77**</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>relapses (cases 4 to 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>5</td>
<td>27.19 ± 9.39</td>
<td>0.07 ± 0.03</td>
</tr>
</tbody>
</table>

a) SR is an indicator for PHA-induced blastogenesis of peripheral blood lymphocytes.
b) Optical density value at 450 nm.
Data represent mean ± S.D.
Significantly different from controls: *P < 0.05, **P < 0.01.

increased to 550 × 10^6 μl. Parasitemia level was extremely low (<0.1%). Case 3 was a 4-year-old male Tosa dog. At the first examination, he developed weakness, pallor of mucous membranes and anoxemia. RBC count was 240 × 10^6 μl. Parasitemia level was low (<1%) for severe anemia. He was treated with DD and thereafter recovered from clinical B. gibsoni infection. Immune response, RBC count and parasitemia level were examined on day 80 post DD administration. RBC count increased to 580 × 10^6 μl. Parasitemia level was 0.6%. As shown in Table 1, the dogs with subclinical B. gibsoni infection (cases 1 to 3) showed lower SR values (p < 0.05) and higher anti-parasite ELISA levels (p < 0.05) than controls. ELISA levels were above an upper negative limit of 0.115 [9]. The higher ELISA levels are consistent with persistent parasitemia regardless of treatment with DD. The lower SR values indicate depressed lymphoproliferative responses.

Case 4 was a 5-year-old female German shepherd. She had a history of recovery from clinical B. gibsoni infection and was complicated with heartworm disease. Clinical signs were weakness, pallor of mucous membranes and anoxemia. RBC count was 170 × 10^6 μl. Parasitemia level reached 22%. Case 5 was a 1-year-old female mongrel dog. She had been diagnosed as babesiosis and treated with DD. On day 20 post DD treatment she suffered from a relapse of clinical B. gibsoni infection, and was referred to University Hospital. At the first examination, she exhibited weakness, pallor of mucous membranes and anoxemia. Blood examination revealed a RBC count of 330 × 10^6 μl and a parasitemia level of 2.5%. Immune response was examined on day 2 post admission. Case 6 was a 3-year-old male poodle. He had a history of repeated relapses of clinical B. gibsoni infection. He suffered from a complication of Hepatozoon canis infection. He developed clinical signs of B. gibsoni infection such as weakness, pallor of mucous membranes and anoxemia. RBC count decreased to 190 × 10^6 μl and parasitemia level reached 4%. As shown in Table 1, the dogs suffering from relapses of clinical B. gibsoni infection (cases 4 to 6) demonstrated prominently lower SR values (p < 0.01) than healthy dogs. Anti-parasite ELISA levels in the dogs of cases 4 to 6 were below an upper negative limit of 0.115 [9], indicating that antibody production was suppressed in dogs suffering from relapses of clinical B. gibsoni infection. These results suggest that immunosuppression occurred in subclinically B. gibsoni-infected dogs by unknown mechanism, thereby resulting in relapses of clinical B. gibsoni infection. Complications, as observed in cases 4 and 6, may also induce relapses of clinical B. gibsoni infection to a certain degree. In case 6 B. gibsoni parasites were resistant to DD, thereby resulting in repeated relapses of clinical B. gibsoni infection. This raises a possibility that changes in the antigenic profile of B. gibsoni parasites, as reported on human malaria by Cox [3], were also involved in relapses of clinical B. gibsoni infection.

It remains to be cleared what induced immunosuppression in B. gibsoni-infected dogs. Complications such as heartworm disease and H. canis infection may possibly play certain roles in induction of immunosuppression. Considering that SR values were depressed even in dogs without any complications (cases 1 to 3 and 5), direct suppressive effects of B. gibsoni parasites on the immune system should be considered. Immunosuppression in haemoprotean infections has been well documented [2, 4, 6–8]. The mechanism by which immunosuppression is induced in haemoprotean infections is not completely elucidated. Cunningham and Kuhn [4] have reported that serum from Trypanosoma cruzi-infected mice directly suppresses the level of responding lymphocytes and, indirectly through the activation of splenic suppressor cells. On the other hand, Wells et al. [12] have reported anti-lymphocytotoxic antibodies in sera of adult Thai people infected with Plasmodium falciparum and P. vivax.

In the present study, immune response was suppressed in dogs with subclinical and clinical B. gibsoni infection. However further detailed study is necessary to clarify the causes of immunosuppression in B. gibsoni-infected dogs and the relationship between immunosuppression and a relapse of clinical B. gibsoni infection. Even the dogs recovered from clinical B. gibsoni infection are at the risk of relapse, so they should undergo follow-up examinations. If any signs of a relapse of clinical B. gibsoni infection are seen, treatment with such drugs as enhance immune response is recommended.

REFERENCES


