NOTES

Inhibitory Effect of Antiserum to Surface Antigen P50 of *Babesia gibsoni* on Growth of Parasites in Severe Combined Immunodeficiency Mice Given Canine Red Blood Cells

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The inhibitory effect of an antiserum to surface protein P50 of *Babesia gibsoni* on the growth of the parasite was determined with severe combined immunodeficiency mice given canine red blood cells. The antiserum to the recombinant P50 protein significantly inhibited the parasite growth, indicating that P50 might be a useful vaccine candidate.

Babesia gibsoni is a tick-borne hemoprotozoan parasite that causes piroplasmiasis in dogs. The disease is characterized by a remittent fever, progressive hemolytic anemia, hemoglobinuria, and marked splenomegaly; in addition, it sometimes causes death (2, 16, 18). *B. gibsoni* infection is endemic in many regions of Asia, Africa, Europe, and the Americas (7, 9). The disease is frequently present in dogs and has recently become a serious clinical problem (1, 3, 11, 14). For the control of *B. gibsoni* infection in dogs, vaccination is generally considered to be the most effective means. It is known that the inactivated whole parasites are useful antigens for vaccination, and they induce partial protection against canine babesiosis (13). However, the quantity and quality of the antigens frequently vary from one batch to another. Furthermore, the production of whole parasites requires that dogs be experimentally infected, which is expensive and time-consuming. The use of recombinant vaccines corresponding to immunodominant antigens of *B. gibsoni* would overcome the problems outlined above.

The surface protein of a parasite can be recognized as a major target by the host immune system during the interaction that takes place between host and parasite. Therefore, a surface protein is a logical target for vaccine production. In previous studies, our group identified a type I transmembrane protein, P50, expressed on the surface of *B. gibsoni* merozoites (5, 6) and demonstrated that the P50 protein was recognized as an immunodominant antigen by the host immune system in dogs infected with *B. gibsoni*. We also reported that the growth-inhibitory effect occurred in *B. gibsoni*-infected severe combined immunodeficiency mice given canine red blood cells

(Ca-RBC-SCID mice) that passively received *B. gibsoni*-infected dog serum (4). Therefore, we assumed that *B. gibsoni*-infected Ca-RBC-SCID mice were potentially useful for determining the growth-inhibitory effect of the antibodies to *B. gibsoni*. In this study, we produced the antiserum to recombinant P50 protein expressed in insect cells and determined its inhibitory effect on the growth of parasites in Ca-RBC-SCID mice infected with *B. gibsoni*.

The expression of a secretory form of a recombinant P50 protein (rP50t) in the culture medium of insect cells infected with a recombinant baculovirus has been described elsewhere (5). The production of antiserum to rP50t (anti-P50 serum) was performed as described previously (12). The control antiserum to a culture medium of insect cells infected with a recombinant baculovirus carrying the *lacZ* gene (anti- β -galactosidase [anti- β -Gal] serum) was also produced. In the Western blot analysis, the anti-P50 serum reacted specifically to a band with a molecular mass of 50 kDa from *B. gibsoni* merozoites but the normal rabbit serum (NRS) or anti- β -Gal serum did not (Fig. 1). In the immunofluorescent antibody test (IFAT) with confocal laser microscopy, the anti-P50 serum reacted strongly to the *B. gibsoni* merozoites collected from a *B. gibsoni*-infected Ca-RBC-SCID mouse, but the NRS or the anti- β -Gal serum did not (Fig. 2). These results indicated that the recombinant P50 protein has a similar antigenic structure as that of the native P50 protein from *B. gibsoni* merozoites and is a useful antigen for the immunization of animals.

The growth-inhibitory effect of the anti-P50 serum on *B. gibsoni* was determined using *B. gibsoni*-infected Ca-RBC-SCID mice. Eighteen female SCID mice (6 weeks old; Clea Japan, Tokyo) were splenectomized as described in a previous paper (4) and divided into three groups of six mice each. Canine RBCs were prepared as described previously (4). Five hundred microliters of a packed cell volume of canine RBCs

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FIG. 1. Western blot analysis of the reactivity of anti-P50 serum against *B. gibsoni* merozoites. Western blot analysis was performed as previously described (5, 10). P50, anti-P50 serum; β -Gal, anti- β -Gal serum. Lanes 1, lysates of *B. gibsoni*-infected RBCs; lanes 2, control lysates of healthy dog RBCs were used for the antigen.

mixed with 500 μ l of anti-P50 serum, anti- β -Gal serum, or NRS (GIBCO BRL, Rockville, Md.) was intraperitoneally injected into the splenectomized mice at 1-day intervals three times (days -6, -4, and -2) before infection with the *B. gibsoni* parasite (NRCPD strain) (5, 8). On day 0, *B. gibsoni*-infected RBCs (5×10^5 per mouse) collected from *B. gibsoni*-infected Ca-RBC-SCID mice were injected intraperitoneally with canine RBCs and one of the sera. The same volume of canine RBCs and one of the sera were injected intraperitoneally at 2-day intervals after infection to maintain the transfusion level of canine RBCs in the peripheral blood and the levels in serum of the mouse. At 0- to 1-day intervals, the peripheral blood was collected from the tail vein and examined for parasitemia by light microscopy of Giemsa-stained thin blood smear films. The parasite proliferation in mice that received the anti-P50 serum was significantly inhibited ($P < 0.05$, from days 5 to 11) in comparison with that in mice that received either the NRS or anti- β -Gal serum (Fig. 3). There was no significant difference between the two groups that received either the NRS or anti- β -Gal serum ($P > 0.2$). The average peak parasitemia in the groups of mice receiving the anti-P50

FIG. 3. Growth-inhibitory effect of anti-P50 serum on *B. gibsoni*-infected Ca-RBC-SCID mice. P50, anti-P50 serum; β -Gal, anti- β -Gal serum. The statistical analysis of the parasitemia was performed with the Student *t* test. Asterisks show the significant differences ($P < 0.05$) between the groups of mice that received the anti-P50 serum and the control groups. The error bars show standard errors of the means.

serum was 1.01%, and the parasitemia remained under 1.12% from day 0 to day 14. In contrast, the average peak parasitemia in groups of mice that received either NRS or anti- β -Gal serum was 6.31% (NRS, $P = 0.0004$) or 5.23% (anti- β -Gal serum, $P = 0.0001$), and the growth curves were similar to those of *B. gibsoni*-infected Ca-RBC-SCID mice in which peripheral RBCs were transfused with phosphate-buffered saline (4). At the peak of parasitemia, the inhibitory effect ratio was 78.5% compared to that of anti- β -Gal serum and 82.1% compared to that of NRS. The titer of the mouse antibody to P50 protein was determined every week by enzyme-linked immunosorbent assay with glutathione *S*-transferase protein as an antigen (5, 6). The titer of the original anti-P50 serum against the glutathione *S*-transferase-P50 protein was 1:51,200 (data not shown) and was maintained at 1:6,400 to 1:12,800 in all

FIG. 2. IFAT analysis of the reactivity of anti-P50 serum against *B. gibsoni* merozoites with confocal laser microscopy. IFAT was performed as previously described (5, 17). The green signals show the expression of P50 protein, and the red signals show the presence of nuclei of merozoites stained with propidium iodide. P50, anti-P50 serum; β -Gal, anti- β -Gal serum.

FIG. 4. Morphological effect of anti-P50 serum on *B. gibsoni*-infected Ca-RBC-SCID mice. P50, anti-P50 serum; β -Gal, anti- β -Gal serum. The upper panels show intracellular parasites, and the lower panels show the presence (or absence) of extracellular parasites.

mice given anti-P50 serum, whereas no antibody to P50 was detected in all mice given NRS or anti- β -Gal serum. The morphology of the *B. gibsoni* parasites was also compared in the groups of mice that received the antiserum (Fig. 4). The *B. gibsoni* parasites showing multiple proliferating parasites (8 to 32 parasites in a single RBC) or extraerythrocytic parasites (4) were detected in the groups of control mice that received either NRS or anti- β -Gal serum. However, significant proliferation of parasites or extraerythrocytic parasites were not detected in the groups of mice that received the anti-P50 serum, and only one to two parasites were detected in single RBCs. These results demonstrated that the antibody to P50 protein significantly inhibited the growth of the *B. gibsoni* parasite in Ca-RBC-SCID mice.

Generally, in a case of intracellular parasite infection, the cellular rather than humoral immune responses are considered to be an important factor for protection against infection. Even though the antibody cannot react to the intracellular parasite, it can react to the pathogen during the extraerythrocytic phase of the parasites. In the present study, the antiserum to the P50 protein inhibited the growth of the parasites, although it did not completely protect Ca-RBC-SCID mice against challenge with *B. gibsoni*-infected RBCs. In natural *B. gibsoni* infection, the parasites were transmitted by tick vector. Thus, further work will be performed in order to determine the protective efficacy against tick-delivered challenge infection. In this study, we determined the parasitemia for only a 14-day infectious period. Therefore, the repeated antiserum infusions might select for resistant parasites that eventually proliferate well. The mechanism for multiple proliferative parasites is still unknown; however, it seems that the lack of humoral immune pressure

leads to the appearance of multiple proliferative parasites, because this form of the parasite was also detected in an in vitro culture of *B. gibsoni* parasites (15). However, the antibody did not react directly with the intracellular parasites. Therefore, it can be hypothesized that the physical stresses or damage caused by the antibody at the extraerythrocytic phase of the parasite might affect any proliferation that might take place in the RBCs, although further studies are needed.

In conclusion, the data here demonstrate that *B. gibsoni* P50 protein has potential as a vaccine antigen for controlling canine *B. gibsoni* infection and that Ca-RBC-SCID mice infected with *B. gibsoni* could be useful as a model for the assay of the inhibitory effect of antibodies to the recombinant protein of *B. gibsoni* on the growth of parasites in vivo. For a follow-up study, it might be worthwhile to immunize dogs with the recombinant P50 protein to test its effectiveness for protection against or ability to combat the clinical symptoms of *B. gibsoni* infection.

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