

Immunization with Recombinant Surface Antigen P50 of *Babesia gibsoni* Expressed in Insect Cells Induced Parasite Growth Inhibition in Dogs

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This is a report of a vaccine trial directed against *Babesia gibsoni* infection in dogs with the use of the recombinant antigen P50. Dogs immunized with P50 showed partial protection manifested as a significantly low level of parasitemia. The results indicated that P50 is a primary vaccine candidate molecule against canine *B. gibsoni* infection.

Babesia gibsoni is a tick-borne intraerythrocytic protozoan parasite that causes piroplasmiasis in wild and domestic canids. The parasite is widely distributed and has been reported from Asia, Africa, Europe, the Middle East, and North America (16). The most common clinical signs of the disease are remittent fever, progressive hemolytic anemia, hemoglobinuria, marked splenomegaly, and, frequently, death (2, 15, 16). Vaccination is generally considered to be the most effective means to prevent or alleviate *B. gibsoni* infection in dogs. However, no vaccine is currently available. Therefore, the development of a vaccine to control canine *B. gibsoni* infection is desired. It has been reported that inactivated whole parasite antigens or inactivated secreted parasite antigens derived from culture supernatant could induce partial protective immunity against canine babesiosis caused by *Babesia canis* (11). However, the production of parasite antigens requires that dogs be experimentally infected, which is expensive and time-consuming, and the quantity and quality of the antigens vary from one batch to another. The use of recombinant antigens would overcome the problems outlined above. In recent studies, vaccine trials against animal babesiosis were mainly focused on the use of recombinant antigens that induce protective immunity (9). A number of different antigens associated with merozoites or merozoite-infected erythrocytes have been identified and applied in vaccine trials. However, their protective efficacies were limited, and vaccines that can induce complete protective immunity have not been developed (1, 9). Thus, further vaccine development study against animal babesiosis has been extremely desired.

In a previous study, we identified a transmembrane protein, P50, which is expressed on the surfaces of *B. gibsoni* merozoites, and we demonstrated that P50 was recognized as an im-

munodominant antigen by the host immune systems of dogs infected with *B. gibsoni* (7). In order to obtain pure and large amounts of P50, we successfully expressed a secretory form of P50 (rP50t) in a culture supernatant of insect cells infected with the recombinant baculovirus by the truncation of the C-terminal anchor region of the transmembrane (6). In a mouse immunization trial, it was confirmed that rP50t retained good immunogenicity (6). Furthermore, we demonstrated that the antiserum against rP50t produced in a rabbit significantly inhibited parasite growth in *B. gibsoni*-infected, canine red blood cell substitution severe combined immunodeficiency (Ca-RBC-SCID) mice (5). These results indicated that rP50t is a useful vaccine candidate for controlling canine *B. gibsoni* infection. In this study, we immunized dogs with rP50t and investigated its immunogenicity and protective efficacy against *B. gibsoni* infection in dogs.

The expression of a secretory form of a rP50t in the culture medium of insect cells infected with a recombinant baculovirus has been described in a previous paper (6). The supernatant containing rP50t was concentrated to 1 mg/ml using Vivapore 10/20 (Vivascience, United Kingdom) and then used in the dogs' immunization trials. A culture medium of insect cells infected with a recombinant baculovirus expressing β -galactosidase (β -Gal) was used as the antigen control. Female specific-pathogen-free beagles (14 to 15 months old) purchased from Chugai Research Institute for Medical Science (Nagano, Japan) were used. Nine dogs were divided equally into three groups. One group was immunized with rP50t. The negative-control group was immunized with β -Gal. The remaining group was used as the nonimmunized control. Dogs in all groups, except for the nonimmunized control group, received four immunizations via the intramuscular route with 500 μ g of antigen mixed with saponin (Q-vac; NOR-VET, Denmark) containing 2 mg of alum (LSL, Tokyo, Japan) as adjuvants at 2-week intervals. Two weeks after the final immunization, dogs were intravenously infected with 2×10^8 *B. gibsoni*-infected RBCs (NRCPD strain) (8) collected from a dog experimentally infected with the *B. gibsoni* parasite (day 0). Figure 1

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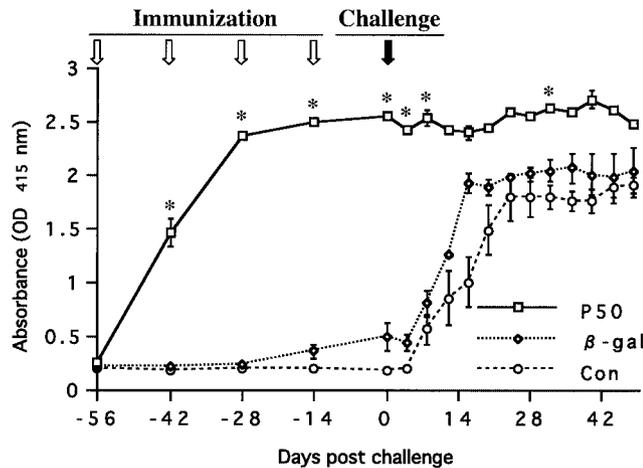


FIG. 1. Determination of antibody response against P50 of dogs immunized with rP50t by ELISA. ELISA was performed as previously described (7). The results are shown as mean values, and error bars represent standard deviations. The statistical analysis was determined by the Student *t* test. Asterisks show the significant difference ($P < 0.05$) between the groups of dogs immunized with rP50t and the control groups. rP50t results are from sera collected from dogs immunized with rP50t, β -Gal results are from sera collected from dogs immunized with control antigen β -Gal, and Con (control) results are from sera collected from nonimmunized control dogs.

shows the specific antibody response in dogs determined by enzyme-linked immunosorbent assay (ELISA) with glutathione *S*-transferase (GST)-P50 as an antigen (4). The immunoglobulin G (IgG) response against P50 was gradually increased after each immunization with rP50t. Two weeks after the final immunization with rP50t, a mean optical density (OD) of more than 2.5 was reached. This OD was significantly higher than the highest OD observed in the control groups after a challenge infection of *B. gibsoni*. The IgG subclasses were also analyzed by the ELISA. There were no significant differences in the IgG1 and IgG2 responses in immunized dogs. In indirect fluorescent antibody test analysis, all sera from dogs immunized with rP50t strongly reacted to the *B. gibsoni* merozoites, but sera from control dogs did not (data not shown). As shown in Fig. 2, the sera from dogs immunized with rP50t specifically recognized the native P50 of *B. gibsoni* merozoites, but the sera

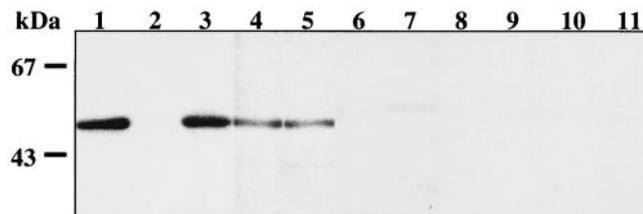


FIG. 2. Western blot analysis of reactivity of sera collected from dogs immunized with rP50t. Western blot analysis was performed as previously described (7). Lanes 1 and 2, monoclonal antibody 31D specific to P50 of *B. gibsoni* (8); lanes 3 to 5, sera collected from dogs immunized with rP50t; lanes 6 to 8, sera collected from dogs immunized with control antigen β -Gal; lanes 9 to 11, sera from nonimmunized control dogs; lanes 1 and 3 to 11, lysates of *B. gibsoni*-infected red blood cells; lane 2, control lysates of healthy-dog red blood cells used as antigens.

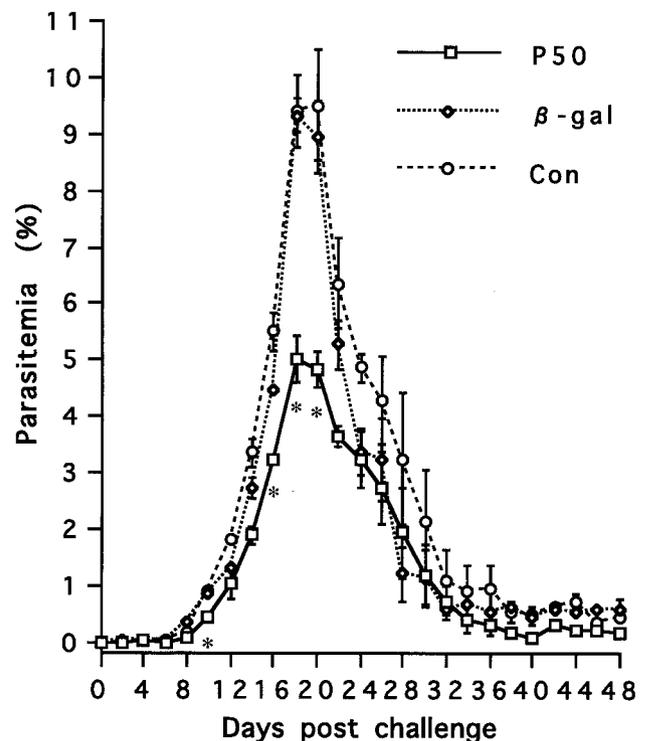


FIG. 3. Parasitemia of dogs after challenge infection with *B. gibsoni*-infected red blood cells. Giemsa-stained thin blood smear films were used for the determination of parasitemia. The results are shown as mean values, and error bars represent standard deviations. Asterisks show a significant difference ($P < 0.05$) between the groups of dogs immunized with rP50t and the control groups. The parasitemias in dogs immunized with rP50t and β -Gal or in nonimmunized control dogs were statistically analyzed by the Student *t* test. rP50t results are from dogs immunized with rP50t, β -Gal results are from dogs immunized with control antigen β -Gal, and Con (control) results are from nonimmunized control dogs.

from control dogs did not. Parasite growth in dogs immunized with rP50t was significantly inhibited ($P < 0.05$ on day 10 and days 16 to 20) in comparison with parasite growth in control dogs immunized with β -Gal or in nonimmunized dogs (Fig. 3). There was no significant difference between the two control groups ($P > 0.2$). At the peak of parasitemia, the ratio of inhibitory effect was 51.2% compared to that of the β -Gal-immunized group and 47.5% compared to that of the nonimmunized group. The packed cell volume of venous blood was measured to determine the anemia level. There were no significant differences in the anemia levels among the three dog groups (data not shown). The subsequent challenge with *B. gibsoni* parasites of the immunized dogs was not performed to confer full protection.

To our knowledge, this is the first report for evaluating the efficacy of a recombinant vaccine against canine *B. gibsoni* infection in dogs. In a previous study, we demonstrated that the antiserum against rP50t inhibited parasite growth in Ca-RBC-SCID mice infected with *B. gibsoni* (5). This result indicated that P50 is a useful vaccine candidate for controlling canine *B. gibsoni* infection. In this study, we demonstrated the vaccine potential of rP50t using dogs, the natural hosts of *B. gibsoni*. Vaccination with rP50t with an adjuvant induced a

high level of specific antibody response against P50 and significant protective immunity against challenge infection with *B. gibsoni* in dogs. This result indicated that P50 is a useful vaccine candidate for controlling canine *B. gibsoni* infection. The nature of this protective efficacy was evident in the significantly low parasite growth rates of immunized animals compared to those of control animals. However, only partial protection was observed in spite of a strong antibody production. The passive transfer of sera from the vaccinated dogs to the nonimmunized dogs and subsequent challenge would provide useful information about the mechanism of the parasite growth inhibition effect induced by the immunization with rP50t. A passive immunization test will be done in the future.

Despite the fact that the dogs vaccinated with rP50t were significantly less susceptible to the effect of parasites, anemia was not significantly prevented in them. This reduction of parasitemia was rather short-term and limited to a very narrow window. Thereafter, there appeared to be little overall protection against effects such as anemia compared to the levels of overall protection of control animals. Therefore, further study of the pathogenesis of canine *B. gibsoni* infection will be necessary to develop a more effective vaccine. In this study, a vaccination of dogs with rP50t induced moderate protection against *B. gibsoni* infection. Thus, further work will be required to improve the protective efficacy. It is known that saponin and alum, used as the adjuvants in this study, mainly stimulate the humoral immune response (3). Generally, in the case of intracellular parasite infection, the cellular immune response is considered to be a more important factor than the humoral response for protection against infection (1). Recently, it was demonstrated that a heterologous prime-boost immunization regimen with a DNA plasmid and a recombinant vaccinia virus, both expressing the same antigen of pathogens, could induce strong immune responses, including cell-mediated immunity (10, 13, 14). In mouse malaria, it has been demonstrated that immunization with such a regimen induced complete protection against sporozoite challenge (12). Therefore, for a follow-up study, we plan to determine the potential use of such a heterologous immunization regimen with P50 to improve the protective effect of P50 against canine *B. gibsoni* infection.

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All experiments described in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

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