Immunization with Recombinant Surface Antigens p26 with Freund's Adjuvants against *Babesia rodhaini* Infection

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(Received 20 May 1999/Accepted 2 March 2000)

ABSTRACT. The surface proteins of *Babesia rodhaini* have previously been shown to induce a high degree of protective immunity. In the present study, one of those proteins, *B. rodhaini* antigen p26 was expressed in *Escherichia coli* and in insect cells infected with a recombinant baculovirus. These proteins were recognized by immune serum from a drug-cured BALB/c mouse. While BALB/c mice immunized with both recombinant antigens and Freund's adjuvants showed 40–100% survival rate against challenge infection with *B. rodhaini*, saponin failed to induce protection, although significant levels of *B. rodhaini*-specific antibodies were produced in both immunized mice (1:1,000–2,000 by indirect immunofluorescent antibody test). The immunization of IFN- γ -deficient mice with the recombinant proteins was not protective against *B. rodhaini* infection, indicating that IFN- γ is one of the important factors for the survival against lethal *B. rodhaini* infection.

KEY WORDS: *Babesia rodhaini*, IFN- γ , mouse, recombinant antigen, survival.

J. Vet. Med. Sci. 62(7): 717–723, 2000

Babesia rodhaini is an intraerythrocytic protozoan parasite which causes a lethal infection in mice. Acute infection can be cured by chemotherapy in mice, the recovered mice being resistant to reinfection [16, 27]. Protective immune responses against *Babesia* infection have been attributed to cell-mediated immunity [6, 9, 28]. In *B. microti* infection, CD4⁺ T cells and IFN- γ play an essential role not only in the resolution of primary infection [9] but also in protective immunity against the challenge infection [8]. Macrophages and T cells are suggested as effector cells for the protective immunity against *B. rodhaini* infection [17, 27]. Recently, CD8⁺ cells have been suggested as major effector cells in the protection against *B. rodhaini* infection [27]. On the other hand, other reports have suggested that CD4⁺ cells especially Th1 cells, are responsible for protection against *B. rodhaini* by inducing cell-mediated immunity [14].

Four antigens of *B. rodhaini* have been identified by monoclonal antibodies. These proteins reacted strongly to the immune serum [18]. The DNA sequences of genes encoding these proteins were also analyzed [18, 20]. Among four antigens, p26 antigen is a merozoite surface protein that has been shown to induce a degree of protective immunity in mice, which is evident both in their increased survival and reduced blood parasitemia [19]. The p26 antigen consists of 337 amino acids, and the molecular masses of precursor and mature proteins are 36.8 K and 34.5 K, respectively [20]. However, the precise role of this protein for protective immunity against the challenge infection is still unknown. In the present study, recombinant p26 antigens of *B. rodhaini* (Br26) were produced in *E. coli* and the baculovirus-insect cell system. The effect of the recombinant antigens on the course of infection with *B. rodhaini* in mice was examined.

MATERIALS AND METHODS

Mice: Female BALB/c mice were purchased from CLEA (Tokyo, Japan). IFN- γ -deficient mice were generated as previously described [22]. Male and female IFN- γ -deficient mice were backcrossed to BALB/c for seven generations and maintained by interbreeding with heterozygous animals, and homozygous (-/-) and wild-type littermates (+/+) were identified as described before [22]. All mice were between 5 and 7 weeks old at the time of the experiment. IFN- γ -deficient mice were housed in filter-topped autoclaved cages and given autoclaved food and water.

Parasite and DNA extraction: Australian strain of *B. rodhaini* was maintained by blood passage as previously described [7]. *B. rodhaini*-infected erythrocytes (70% parasitemia) were washed with phosphate buffered saline (PBS), lysed in 0.1 M Tris-HCl (pH 8.0) containing 1% SDS, 0.1 M NaCl, and 10 mM EDTA, and then treated with proteinase K (100 μ g/ml) for 2 hr at 55°C. The genomic DNA was extracted by phenol-chloroform extraction and precipitated by ethanol. The pellets were resuspended in distilled water and stored at 4°C until use.

Expression of recombinant Br26 in *E. coli*: DNA encoding p26 was amplified by polymerase chain reaction (PCR) from *B. rodhaini* genomic DNA using two oligonucleotide primers with *Bam* HI sites, based on a published sequence for the p26 antigen gene from the Antwerp strain [18]. PCR cycles consisted of an initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, annealing at 63°C for 2 min, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The amplified DNA was loaded on to 1.5% agarose gel electrophoresis. For expression in *E. coli*, a DNA band of the expected size was recovered from the gel

and ligated into the *Bam* HI site of the expression vector pGEMEX2. The ligation mix was transformed into competent *E. coli* DH5 α cells, and colonies of transformants were selected on LB agar containing 50 μ g/ml ampicillin. Plasmid DNA was isolated and checked for the presence of *B. rodhaini* p26 gene in the correct orientation using appropriate enzyme digests. Plasmid DNA (pGEMEX/Br26) from a selected colony was purified and used to transform *E. coli* JM109 (DE3) for the expression of p26 as a fusion protein of the bacteriophage T7 gene 10 protein according to the manufacturer's instructions (Promega, U.S.A.). Transformed *E. coli* were grown at 37°C in 75 cm² culture flasks, induced with isopropylthio- β -D galactoside (IPTG), and then harvested by centrifugation. *E. coli* were from one culture flask suspended in one ml of PBS and lysed with lysozyme followed by ultrasonic treatment and used as crude recombinant antigen for immunization of five mice.

Expression of recombinant Br26 using baculovirus: For expression using baculovirus, amplified DNA by the PCR was ligated into the *Bam* HI site of baculovirus transfer vector pBacPAK8, and a recombinant transfer vector pAK/Br26 was isolated. Purified plasmid DNA was mixed with wild-type baculovirus DNA (Bsu36I-digested linear AcNPV) and transfected into *Spodoptera frugiperda* (Sf9) cells in TC-100 insect medium (GIBCO BRL, U.S.A.) supplemented with 10% fetal calf serum and 0.26% Bacto tryptose broth (Difco, U.S.A.) using the lipofectin reagent (GIBCO BRL, U.S.A.). After 4 days of incubation at 27°C, the culture supernatant containing recombinant virus was harvested and subjected to plaque purification. The expression of Br26 in plaques was checked by the indirect immunofluorescent antibody test (IFAT) using mouse anti-*B. rodhaini* serum, and a positive plaque was picked. After three cycles of purification, a recombinant baculovirus (AcBr26) was obtained. This was then amplified in a Sf9 cell culture in 75 cm² culture flasks and harvested by centrifugation. Infected Sf9 cells from one culture flask were suspended in one ml of PBS and freeze-thawed three times and stored at -80°C.

Analysis of recombinant antigens: Recombinant antigens in *E. coli* or the baculovirus-insect cell system were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot.

Indirect immunofluorescent antibody test (IFAT): IgG antibody titers were determined by the IFAT using *B. rodhaini*-infected erythrocytes as described previously [26].

Immunization and challenge infection: Recombinant antigen in *E. coli* or baculovirus-infected Sf9 cell lysates was used for immunization of five mice of each group with 0.01% saponin or Freund's adjuvant. Five BALB/c or IFN- γ -deficient mice were immunized two or three times subcutaneously (saponin) or intraperitoneally (Freund's adjuvants) with 0.2 ml of *E. coli* expressed protein or baculovirus-infected Sf9 cell lysates at 2-week intervals. When Freund's adjuvant was used, the primary immunization was performed with Freund's complete adjuvants and later immunizations were carried out with Freund's incomplete adjuvant.

The Australian strain of *Babesia rodhaini* was used for the

challenge experiments [7]. Mice (four or five mice/group) were challenged with 1×10^3 or 1×10^4 parasitized erythrocytes 2 weeks after the last immunization. Parasitemias in between 200 and 1×10^4 erythrocytes were monitored by microscopic examination of Giemsa-stained thin smears of tail blood prepared every 2 days.

Statistics: Fisher's exact test and Student's *t* test were used to determine the significant difference in the survival rate and in the parasitemias of mice between the immunized and control groups, respectively.

RESULTS

Expression of Br26: P26 antigen gene was amplified by PCR from genomic DNA of Australia strain of *B. rodhaini*. The PCR product of P26 antigen gene from Australian strain was smaller than those from Antwerp strain and consisted of 913 base pairs DNA and 80% homology was observed with p26 of Antwerp strain of *B. rodhaini*. SDS-PAGE analysis of recombinant Br26 expressed in *E. coli* showed a major band with a molecular weight (MW) of 67 kDa and one with a MW of 36 kDa in the T7 gene 10 protein used as a control (Fig. 1A). This fusion recombinant protein was recognized by mouse *B. rodhaini* immune serum (Fig. 1B, lane 1), but the control T7 gene 10 product was not (Fig. 1B, lane 2). Recombinant Br26 expressed in Sf9 cells was not observed as a major band by SDS-PAGE analysis, but immunoblotting analysis detected a major band reacting with the immune serum against *B. rodhaini* in Sf9 cells with a molecular weight of 33 kDa (Fig. 1B, lane 3) as well as the lysate of *B. rodhaini*-infected erythrocytes (Fig. 1B, lane 5).

Antibody responses after immunization with recombinant Br26: Antibody response induced by immunization of five mice with recombinant antigens plus saponin or Freund's adjuvant were determined using serum samples collected before and after the challenge infection. After the second immunization, high specific IgG antibody titers (1:1,000–2,000 by IFAT) were detected in all five mice immunized with both recombinant antigens regardless of the adjuvants. However, no significant increase of antibody titers were found after third immunization of recombinant antigens. Similar high specific IgG antibody titers were observed one week after challenge infection. In contrast, IgG antibody titers were not detected in the control groups after the second immunization, and very low titers were seen in five mice of the control groups after the challenge infection.

Immunization trials: In a preliminary experiment, the immunizing efficacy of recombinant baculovirus-infected Sf9 cell lysate was assessed with two different adjuvants, saponin and Freund's adjuvants (Fig. 2). When saponin was used for the immunization of mice, five mice immunized three times with both control and Br26 containing cell lysates died with the average peak parasitemia reaching 71.6% and 73.1% respectively within 12 days following the challenge infection with 1×10^4 parasites. In contrast, when Freund's adjuvant was used, mice immunized three times with cell lysates containing Br26 showed a higher survival rate (60%) against

Fig. 1. SDS-PAGE analysis of recombinant Br26 antigen expressed in *E. coli* and Sf9 cells. (A) Coomassie blue-stained gel. (B) Immunoblot probed with hyperimmune mouse serum against *B. rodhaini*. Lane 1, Br26 expressed in *E. coli*; lane 2, T7 gene 10 protein; lane 3, Br26 expressed in Sf9 cells; lane 4, Sf9 cells infected with wild-type baculovirus; lane 5, *Babesia rodhaini*-infected erythrocytes; lane 6, uninfected mouse erythrocytes.

lethal infection with *B. rodhaini* compared to mice immunized with the same recombinant antigen and saponin. However, there was no statistically significant difference between the group immunized with recombinant antigen plus Freund's adjuvants and the control group inoculated with Freund's adjuvants only, which showed a 40% survival rate. The parasitemias from both groups immunized with Freund's adjuvants were lower than those from the two groups immunized with saponin. Therefore, Freund's adjuvant was used for further experiments.

Three immunizations of mice with recombinant Br26 expressed in *E. coli* also resulted in a higher survival rate against *B. rodhaini* infection in BALB/c mice (Fig. 3). Although one of the four mice immunized three times with pGEMEX/Br26 with Freund's adjuvants died, the three surviving mice showed a 100% survival rate against challenge infection. Control mice immunized with T7 gene 10 protein showed a 25% survival rate against the *B. rodhaini* challenge infection.

Because there was not much difference in antibody responses after the second and third immunizations, five mice were challenged with 1×10^4 *B. rodhaini* after the second immunization (Fig. 4). Five naive control mice immunized with PBS emulsified with Freund's adjuvants died 10–12 days after the challenge infection with an average peak parasitemia of 83.4%. Mice immunized with recombinant Br26 expressed in *E. coli* showed a higher survival rate (60%) compared to the naive control. Mice immunized with recombinant baculovirus-infected Sf9 cell lysates showed a 40% survival rate, but the difference was not statistically significant compared to naive control. In the mice there was a slow increase in para-

sitemia and a significantly lower peak parasitemia compared to that of naive control mice. Mice immunized twice with Freund's adjuvants plus T7 gene 10 protein or Sf9 cells infected with wild type baculovirus showed a 20 % survival rate.

The course of infection of B. rodhaini in IFN- γ -deficient mice: Five IFN- γ -deficient or BALB/c mice of each group were immunized twice with recombinant Br26 expressed in *E. coli* or in recombinant baculovirus-infected Sf9 cells emulsified with Freund's adjuvants. In these mice there were no apparent adverse reactions to the immunizations and they were challenged with 1×10^3 *B. rodhaini*. All five IFN- γ -deficient mice of four experimental groups succumbed on days 8 to 13, showing higher parasitemia more than 80% (Fig. 5), whereas BALB/c mice showed a lower parasitemia and survival rate in mice immunized with recombinant Br26 antigen expressed in *E. coli* or in recombinant baculovirus-infected Sf9 cells, respectively as shown in Fig. 4. These results suggest that IFN- γ is one of the important factors for the survival against lethal *B. rodhaini* infection.

DISCUSSION

In the present study, recombinant antigens of *B. rodhaini* p26 have induced partial survival against lethal challenge infection with *B. rodhaini* when mice were immunized with Freund's adjuvants. The molecular weights of these recombinant antigens expressed in Sf9 cells transfected with recombinant baculovirus or in *E. coli* were smaller than those of naive antigen. The difference of molecular weight between naive and recombinant antigens may be explained by the difference in parasite strains, the antigenic variation [2, 12], or the lack of

Fig. 2. Comparison of parasitemia and survival rate in mice challenged with *Babesia rodhaini* after immunizations with recombinant Br26 antigen expressed in Sf9 cells transfected with recombinant baculovirus. Five mice were immunized three times with Br26 plus saponin or Freund's complete and incomplete adjuvants (FA) at two-week intervals and were challenged with 1×10^4 *B. rodhaini*. The graphs show the average parasitemia (A) and the survival rate (B).

glycosylation of recombinant antigen. It should be noted that the gene sequence coding for the p26 was originally obtained from the Antwerp strain [16], whereas the genomic DNA used for PCR in the present study was obtained from the Australian strain. In spite of the smaller molecular weight of recombinant antigens, they were recognized by the immune serum from mice recovered by the chemotherapy. Our results indicate that the recombinant Br26 protein shares immunologically active epitope with naive p26.

In rodent malaria infection, recombinant antigen was used for the immunization of mice and Freund's adjuvant was more effective than saponin for the induction of protective immunity [3]. In the present study, the combination of recombinant

Fig. 3. Comparison of parasitemia and survival rate in mice challenged with *Babesia rodhaini* after immunizations with recombinant Br26 antigen expressed in *E. coli*. Four mice were immunized three times with either Br26 antigen expressed in *E. coli* or T7 gene 10 protein as a control with Freund's complete and incomplete adjuvants (FA). One mouse died after the second immunization. Two weeks after immunization, the mice were challenged with 1×10^4 *B. rodhaini*-infected erythrocytes. The graphs show the average parasitemia (A) and the survival rate (B).

antigens and Freund's adjuvants induced a partial survival. Freund's adjuvants are known to induce a strong cell-mediated immunity as well as an antibody response in rodent malaria infection [3, 4]. Interestingly, immunization of mice with Freund's adjuvants could induce the partial survival against lethal *B. rodhaini* infection and this effect may be explained by the nonspecific activation of macrophages. Fur-

Fig. 4. Comparison of parasitemia and survival rate in mice challenged with *Babesia rodhaini* after two immunizations with recombinant Br26 antigens. Five mice were immunized twice with recombinant Br26 protein expressed in *E. coli* or in Sf9 cells transfected with recombinant baculovirus at two-week intervals. The control mice were immunized with T7 gene 10 protein or Sf9 cells infected with wild-type baculovirus with the same schedule. Mice were challenged with 1×10^4 *B. rodhaini* infected erythrocytes two weeks after the last immunization. The graphs show the average parasitemia (A) and the survival rate (B).

thermore, nonspecific protective response against lethal infection with *B. rodhaini* may also be explained by the facts that immunization with complete Freund's adjuvants induced Th1 type cytokine responses and immunization with saponin induced Th2-type cytokine responses in *Trypanosoma cruzi* or ovalbumin antigenic stimulation [21].

In the earlier study [19], immunization with naive, affinity-

Fig. 5. Effect of immunization with Br26 antigen in IFN- γ deficient mice. Five IFN- γ deficient mice were immunized twice with recombinant Br26 antigens expressed in *E. coli* and Sf9 cells transfected with recombinant baculovirus. They were then challenged with 1×10^3 *B. rodhaini*. The graphs show the average parasitemia (A) and the survival rate (B).

purified p26 antigen induced a degree of protective immunity in mice when saponin was used as an adjuvant. Saponin is a glycoside derived from plants and used as an adjuvant in vaccination against protozoan infections [10, 13, 23, 24]. In the present study, however, mice immunized with recombinant antigens in the presence of saponin failed to induce a protective response against lethal challenge infection. The lack of protection in mice immunized with saponin was not due to the failure to respond to intact parasite antigens, since the antibody response to infected erythrocytes in immunized mice with saponin was comparable to that of immunized mice with Freund's adjuvants. Therefore, our results indicate that the antibody response did not correlate the protection against the challenge infection, and cell-mediated immunity rather than humoral immunity is involved in the mechanism behind the

increased survival of mice immunized with Br26 recombinant antigen against the challenge infection with *B. rodhaini*.

Failure of IFN- γ -deficient mice to induce protective immunity against challenge infection provided further evidence that cell-mediated immunity is very important for the protection induced with the recombinant antigens. The importance of IFN- γ in the protective immunity has been suggested in *Babesia* [5, 8, 14] as well as other intracellular protozoan infections such as *Plasmodium*, *Toxoplasma*, and *Trypanosoma* [1, 11, 15, 25]. Further studies on IFN- γ as well as other cytokine responses such as TNF- α , IL-10 and IL-12, and studies on other antigens and adjuvants enhancing specific immune responses will deepen the understanding of the immune mechanism and stimulate the development of vaccines against the *Babesia* infection.

ACKNOWLEDGMENTS. This work was supported by Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science, Culture and Sports of Japan.

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