Immunological Changes in *Babesia rodhaini* Infected BALB/c Mice after Treated with Anti-Babesial Drug; Diminazene Diaceturate

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**ABSTRACT.** A model system capable of investigating immunological changes was first established in *Babesia rodhaini* infected mice with an aid of a drug, diminazene diaceturate (DD). Intraperitoneal (ip) inoculation with *B. rodhaini* resulted in acute death in euthymic (nu+/+) and athymic (nu/nu) BALB/c mice. Treatment with DD at an early stage of infection saved both mice from acute death. Parasitemia recurred in some of them but resulted in death only in nu/nu mice. A re-challenge with 10⁵ parasitized erythrocytes (PE) on the surviving mice on day 28 post infection revealed resistance in nu/+ but not in nu/nu mice. The results suggested a participation of the thymus in the protective mechanisms. Immunological changes were then observed on nu/+ and nu/nu mice which were inoculated ip with 10⁶PE and treated with the drug, and then challenged with 10⁵PE ip on day 28. An antibody response was measured with immediate reaction by footpad injection of a soluble antigen of *B. rodhaini* and by ELISA of serum antibody using the antigen and protein A, on day 10 and later, and further a pronounced response was detected after re-challenge in nu/+ mice. No response was detected by ELISA in nu/nu mice. Delayed footpad reaction was seen in nu/+ mice by day 14 and later but it was suppressed after the re-challenge. When spleen cells obtained from drug-treated and re-challenged nu/+ mice on day 8 after the re-challenge were transferred to nu/nu mice and the nu/nu mice were infected with 10⁵PE ip, 3 out of the 5 recipient mice survived and showed a low level of transient parasitemia, whereas remaining 2 mice died in a short period of infection accompanying severe parasitemia. The model system seems make an analysis of effective cells in babesial immunity feasible.—**KEY WORDS:** *Babesia rodhaini*, BALB/c mouse, diminazene diaceturate, immune cell transfer, nude mouse.

Babesiosis is an important haematoproteozoan disease affecting various kinds of domestic animals in the world and results in a substantial economic loss in several important animal species. Babesiosis in these natural hosts is characterized by a precise balance between the parasite's and the host's reactions elicited by the infection [1]. The endeavour to analyse immunological events in this host-parasite relation, which is a prerequisite to develop the control measure of this pathogen, has developed the laboratory animal models such as *Babesia rodhaini* and *B. microti* in the mouse and rat [1, 2, 6]. The importance of immunized T cells which were thought to act in cooperation with specifically sensitized B cells and to participate in immunological memory has been suggested by investigations using adoptive transfer of immunity with lymphocytes from immune donors [3, 4, 7, 11, 14]. However, the precise nature of cellular interactions leading to immunity still remains to be elucidated. In most of these studies, immune cells were prepared from hosts with a non-lethal docile infection: either from *B. rodhaini*-infected rats [7] or from *B. microti*-infected mice [3, 4, 11], both of which recovered spontaneously from the infection. We attempted to explore the adoptive immunity using a host-babesia model showing more severe infection. As far as we know only one successful experiment has been reported in mice with *B. rodhaini* [14]. *B. rodhaini* infection in the mouse is uniformly lethal [1], and therefore, it has been believed that this animal model is not suitable for the immunological study [1]. However, it was shown repeatedly that drug treated *B. rodhaini*-infected mice showed resistance [2, 5, 8, 10, 14], indicating a very effective immunity left after the drug treatment. The present report describes immunological changes in infected and drug-treated BALB/c mice which carry nu gene heterozygously (nu+/+) or homozygously (nu/nu) and successful transfer of immunity with spleen cells from drug-cured and re-challenged nu/+ mice to infected nu/nu mice.

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MATERIALS AND METHODS

Mice: Female nu/+ (euthymic) and nu/nu (athymic) mice of BALB/c background were supplied by the Breeding Unit of Nihon Institute of Biological Science Co. (NIBS, Tokyo) at 8 weeks of age. Nu/+ mice were kept in plastic cages with corn tips (Okazaki Sangyo, Tokyo) as a bedding material and given pellets (NMS, NIBS, Tokyo) and tap water ad libitum. Nu/nu mice were bred in a clean room and given sterilized pellets and tap water ad libitum.

Babesia organisms: The Australian strain of Babesia rodhaini was obtained from the Kyushu Branch of the National Institute of Animal Health, Japan in 1980, and maintained by a weekly blood transfer to BALB/c mice (NIBS).

Infection of mice and observation: Babesia donor mice were exsanguinated at the peak of parasitemia and the number of parasitized erythrocytes (PE) was counted on Diff-Quik (Kokusai Shiyaku, Kobe) stained blood preparation. Infected blood appropriately diluted with physiological saline was used as an inoculum. Properly diluted PE suspension was injected intraperitoneally (ip) in a volume of 0.05 ml per mouse. Mice were periodically (usually daily) checked for the rate of PE in blood obtained from the tail vein, and also observed for clinical signs until death or killed during the experiment.

Drug and administration: Diminazene diaceturate (DD, Ganaseg, Nihon Squib, Tokyo) was dissolved in sterilized distilled water at a concentration of 50 mg/ml, and used within 48 hrs after preparation. An amount of 0.1 ml was daily injected ip or intramuscularly (im) at the thigh (approximately 20 mg/kg body weight) for three consecutive days. It was confirmed previously and in this experiment that this regimen was enough to induce a decline in PE, provided the drug was commenced to be injected well before the terminal stage of infection.

Babesia rodhaini antigen: Infected blood containing 65% PE was dissolved in phosphate-buffered saline (PBS) and treated by a ultrasonicator at 15 KHz for 15 min (Fuji Electroical Inst., Tokyo). After centrifugation at 7,000 G, the sediment was kept in a freezer at -20°C and used as antigen (S antigen, Saeki, H., in Dissertation for Ph.D. Degree, The Nippon Veterinary and Zootecchnical College, 1986) for ELISA as well as for footpad testing.

Antibody measurement: Test sera were diluted 1:100 in PBS containing 1% bovine serum albumin (Biocell Laboratories, CA.). Fifty μl of the sera was placed into each well on polyvinyl chloride microtiter plates (Falcon Plastics, CA.) coated with S antigen (0.5 μg protein/well). After incubated for 1 hr at 37°C, the plates were washed with PBS containing 0.5% Tween 80 (Tokyo Kasei, Tokyo), and 25 μl protein A conjugated with horse-radish peroxidase (Cappel, Durham) was added to each well. The plates were incubated for 10 min at 37°C and then washed again. Then, 50 μl of freshly prepared enzyme substrate, consisting of 0.8 mg 2,2-azino-di-[3-ethyl-benzthiazolin sulfonic acid] (ABTS, Boehringer Mannheim, West Germany)/ml and 20 μl of 0.3% hydrogen peroxidise/ml in 0.1 M citrate buffer (pH 4.0), was added to each well, and the plates were allowed to stand for 30 min at room temperature under subdued light. The absorbance of the reacted solution was measured by a spectrophotometer (MPR A4 Toso, Tokyo) at 405 nm.

Footpad reaction: Antigen solution at a concentration of 80 μg/ml of PBS was intradermally injected into the right footpad at 0.05 ml per foot while the same amount of PBS was injected into the left foot of the same mouse. Three (immediate) and 24 hr (delayed) later, the thickness of the feet was measured dorso-ventrally with a caliper (Mitsutoyo, Tokyo). The increased footpad thickness was obtained by subtracting the thickness of left foot from that of the right.

Schedule of treatment and immunological testing: The schedule of treatment and immunological testing employed is illustrated in Fig. 1.

Batches of nu/+ or nu/nu mice were ip challenged with 10^4 PE and then treated with DD for 3 consecutive days from day 6 or 7 after infection, when parasitemia did not reach 10%. After infection, 2 nu/+ and 2 nu/nu mice each were randomly sampled for immunological tests every 3 or 4 days until day 21. Nu/+ mice were re-challenged with

![Fig. 1. Schedule of drug-treatment and re-challenge. Immunological test was made for serum antibody and footpad reaction. Open arrow indicates the day of transfer.](image-url)
10⁶ PE ip on day 28 and sampling was continued until day 42. The mice sampled were tested for footpad reaction with S antigen and then killed on the day next to obtain sera for antibabesial antibody titration. Nu/nu mice were bled to get sera at the same time as in nu/+ mice but footpad test was not done.

Spleen cell transfer: Infected and treated mice were used as immune cell donors 8 days after re-challenge (See Results). Intact mice were used as normal cell donors. The spleen was aseptically removed under ether anesthesia, and single cell suspension was made by filtrating the cells through a stainless sieve into culture fluid, RPMI 1640 (Gibco, New York) supplemented with 2 mg/ml L-glutamine, 5x10⁻⁵ M 2-mercaptoethanol, 20 µg/ml gentamycin and 10% fetal bovine serum (Immunobiology Lab., Gunma). After disrupting of erythrocytes by pulse treatment with Tris ammonium chloride for 3 min on ice, cells were suspended in culture solution, and, after washed by low centrifugation, nucleated cells were counted by the trypan blue dye exclusion method. A dose of 10⁶ spleen cells was injected ip into each recipient nu/nu mice, and on the next day they were inoculated with babesia parasites.

RESULTS

Susceptibility of BALB/c (nu/+ and nu/nu) mice to B. rodhaini: Groups of nu/+ and nu/nu mice consisted of 5 or 7 animals each. Each group of mice was ip inoculated with 3 doses of B. rodhaini-parasitized erythrocytes, and parasitemia and death in each group were recorded daily (Fig. 2). All the mice died by day 17 post infection and the average survival time was in response to the dose. No marked difference was seen in the development of parasitemia between nu/+ and nu/nu mice, however it was noted that a few nu/nu mice, 1 in 10⁶ group and 2 in 10⁴ group, exceptionally showed a prolonged clinical course before death.

Clinical course and parasitemia after treatment with DD: In four groups of nu/+ or nu/nu mice, A, B, C, and D, inoculated with 10⁶ PE ip, the treatment with DD was started for subgroups of 5 mice each with different levels of parasitemia: less than 10% (subgroup 1), 10–20% (subgroup 2), 20–30% (subgroup 3) and more than 30% (subgroup 4). The commencement of treatment approximately corresponded to the time after infection. Since both nu/+ and nu/nu mice suffered from very acute infection, the number of mice available at the higher parasitemia levels was reduced, because they died before the treatment started. The number of mice used in this experiment and their fate after treatment are shown in Table 1. The administration route of DD was ip and im, but there was no marked difference between both routes in effectiveness. Subgroup 1 was prevented from acute death, in which treatment was started at an early stage of infection, while subgroup 4 was not. The preventive effect was moderate in subgroup 2 and 3. There was no difference in the preventive effect of acute death between nu/+ and nu/nu mice. In both mice the level of parasitemia started to decrease on day 3 of treatment forming similar declining curves (data not shown). In some mice, however, prevented from acute death by treatment, 4 out of 24 nu/+ and 12 out of 20 nu/nu mice, parasitemia recovered usually around 7 to 10 days after the primary parasitemia subsided. The four nu/+ mice with recurred parasitemia recovered again, while all the 12 nu/nu mice eventually died following increasing levels of parasitemia.
Table 1. Clinical course after treatment with diminazene diaceturate

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. mice in acute phase</th>
<th>Fate of mice with recurred parasitemia</th>
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<tr>
<td></td>
<td>No. mice</td>
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<td>A-1</td>
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<td>A-2</td>
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<td>B-2</td>
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<td>B-3</td>
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<tr>
<td>B-4</td>
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<td>C-1</td>
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<td>C-4</td>
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<td>D-1</td>
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<td>D-2</td>
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<tr>
<td>D-3</td>
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<tr>
<td>D-4</td>
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\[ \text{duration of parasitemia} \]
\[ \text{days post infection} \]

\(16-29\)  
\(19-25\)  
\(14-22\)

\(15-23\)

\(13-18\)
\(15-20\)
\(16-23\)
\(17-23\)

\(13+\)
\(14-19\)
\(14-21\)
\(15-20\)
\(15-21\)
\(16-23\)
\(18-17\)
\(14-24\)

\(11-19\)

\(a\) Groups A through D are each divided into 4 subgroups, 1-4, according to parasitemia when treatment was started; 1:<10%, 2:10-20%, 3:20-30%, 4:>30%.

\(b\) Route of injection of diminazene diaceturate: im, intramuscular; ip, intraperitoneal.

\(c\) Died without parasitemia.

Re-challenge of the drug-cured mice: Some surviving mice in subgroup 2 and 3, which remained negative for parasitemia up to day 21 post infection, that is, 10 to 18 days after treated with DD, were re-challenged with \(10^5\)PE at day 28 and were observed whether parasitemia recurred (Fig. 3). In all the 12 nu/+ mice, parasitemia was noted on day 3-4 post-rechallenge and reached a peak 3-4 days later, and then subsided by day 10 post-challenge. One mouse died exceptionally with increasing parasitemia (data omitted from Fig. 2). In the 6 nu/nu mice, parasitemia appeared on day 5 post-rechallenge and the level increased gradually with marked fluctuations, and all the mice died by day 21 post-rechallenge.

Immunoological changes in infected, drug-treated and re-challenged mice: Since the above described experiments showed that drug treated nu/+ mice can recover from the acute babesial infection and exhibit resistance to re-challenge, we examined immunological responses of the drug-cured mice.

Fig. 3. Development of parasitemia in drug-treated and surviving nu/+ (○, □, △) and nu/nu (▲, Δ) mice after re-challenge with \(10^5\)B. rodhaini. Level of parasitemia after re-challenge in 2 to 4 mice of the groups shown in Table 1: A-2(○), A-3(□), B-2(▲), B-3(△), C-2(▲), and D-2(△). S.D.'s are not indicated.
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Fig. 4. Development of parasitemia (top) and immune response (middle and bottom) after infection followed by drug-treatment and re-challenge. The protocol of experiment is shown in Fig. 1. Level of parasitemia (top) in nu/+ (clear bar) and nu/nu (black bar). Footpad swelling with S.D. in nu/+ mice (middle) at 3 hr (clear bar) and 24 hr (hatched bar) after infection. Serum antibody (bottom) in nu/+ (---) and nu/nu (--O--) mice. 2 mice in each group. Open arrow indicates the day of re-challenge.

All the treated mice were checked for parasitemia before killed.

Results of the footpad reaction are shown in the middle column of Fig. 4. The immediate reaction appeared on day 10 and remained positive, except on day 17, throughout the experimental period including that after re-challenge. The delayed swelling reaction became apparent on day 14 and remained positive up to day 28 when re-challenge was made. After re-challenge, the delayed swelling reactions turned out almost negative.

Antibody response was examined on sera of both nu/+ and nu/nu mice (bottom column in Fig. 4). Positive reaction appeared in nu/+ mice by day 7 or 10 and reached the first peak on day 14, then declined on day 17. After that the titer gradually increased to reach the second peak on day 28. After re-challenge, very high titers were seen in all the cases except on day 31, that is, 3 days after re-challenge, when one of the two mice showed a relatively low titer. No positive cases were seen in nu/nu mice.

Parasitemia, shown at the top column of Fig. 4, showed an expected level. In nu/+ mice transient parasitemia was seen on days 10 and 14, and reappeared on day 38, 10 days after re-challenge. Nu/nu mice also responded to the drug, and parasitemia subsided by day 14. But, it recurred on day 21 and reached a high level on day 24. Two nu/nu mice survived day 21. One of them died on day 28 and the other was killed on day 28 (data not shown).

Adoptive transfer of immunized spleen cells to nu/nu mice infected with B. rodhaini: Whether immunity observed in the drug-treated nu/+ mice as shown in the previous experiments can adoptively be transferred to native mice or not was examined using spleen cells of re-challenged mice as a donor. Batches of nu/+ mice were infected ip with 10^6 PE and treated with the drug as described before. Surviving mice were re-challenged on day 28 with 10^6 PE ip (see Fig. 3) and the spleen was removed 8 days after re-challenge. Nu/nu mice were used as recipients. Five nu/nu mice received 0.5 ml of the immune spleen cell suspension including 10^6 spleen cells ip. The same amount of spleen cells from
normal mice was injected into 5 other nu/nu mice. Other five nu/nu mice were left as untreated controls. On the next day, all groups were challenged with 10⁴PE and parasitemia and death were recorded daily. As shown in Fig. 5, two mice that had received immune spleen cells died in the early period but the remaining 3 survived the end of experiment on day 17. Mice that had received normal spleen cells died successively, the last one on day 17. Mice without cell transfer died by day 13. All the dead mice including those which received immune spleen cells developed high level of parasitemia prior to death, but surviving immunized cell recipients exhibited only a low level of transient parasitemia.

DISCUSSION

In the present experiments clinical changes, including development of parasitemia, in euthymic and athymic mice treated with DD suggested that the thymus does play an important role in subsidence of recurring parasitemia, and the re-challenge experiments further confirmed that the resistance to re-challenge developed under the presence of the thymus.

Although drug-treated mice have been frequently used in the immunological investigation of babesial infection [2, 5, 7-9, 14], there seems to be no analytical study on the immunological phenomena which occur following the treatment. The results obtained with euthymic mice in the present study indicated clearly that antibody response as well as cell mediated immunity developed after the drug administration. Both antibody level and cell mediated immunity, the latter of which is exhibited by delayed footpad reaction, reached a peak on day 14, shortly before subsidence of parasitemia. Antibody level shown by both ELISA and immediate footpad reaction dropped on day 17 but no marked change was seen on the delayed footpad reaction. The antibody level decreased with the termination of parasitemia. This decrease may be explained by releasing a large amount of antigen after babesial organisms are killed. After the re-challenge, antibody response was markedly boosted after a transient drop on day 3 post re-challenge while delayed footpad reaction was profoundly suppressed at least by day 14 post re-challenge. It is of interest that antibody response developed in reverse to cell mediated immune response after re-challenge. Suppression of the delayed-type hyper-sensitivity to babesial antigen by prior infection with babesia organisms has been reported [12, 13]. Many speculative consideration may explain the immunological features observed, however, more precise analysis of cellular and antibody class changes seems to be important to understand this phenomenon more accurately.

Adoptive immunity is a conventional method to identify the effective cell population which confers immunity in vivo. The preliminary experiments using the drug-treated mice infected with B. rodhaini showed that immune spleen cells obtained from mice on day 8 after boosting by re-challenge with the parasite can adoptively transfer immunity against B. rodhaini to athymic nude mice. Thus, analysis of the effective cell(s) in this adoptive immunity will become feasible. This adoptive transfer of immunity, however, was not uniformly established in recipient nude mice since 2 out 5 recipient nude mice suffered from a very acute babesial infection. An accelerating effect of infection by T cells has been suggested by several authors [5, 10] in murine babesial infection. This phenomenon might suggest the importance of cellular interaction to regulate immunity, because in the present experiments unpurified spleen cells were transferred and so various populations of T and B cells and other lymphoid cells would be present among the donor cells. Protection of mice by pretreatment with cyclophosphamide from death by B. rodhaini infection [14] also implies that a precise balance of lymphoid cells influences the efficacy of immunity. On the other hand, the fact that nu/nu mice exceptionally developed a prolonged course of infection in contrast to nu/+ mice which died uniformly of an acute infection might suggest that a non-immunological defence mechanism(s) also plays a role in the defence mechanism of babesia infection in the mouse. Further, the result that parasitemia was more delayed in nu/nu drug-treated mice after re-challenge infection than in the primary infection and also in re-challenged nu/+ mice also implies the presence of another thymus-independent mechanism(s) which interferes with babesial multiplication although it does not seem so effective to control the infection.

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