バベシア感染マウス新生仔における Babesia rodhaini 感染に対する抵抗性

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著者	桜井, 治久 高橋, 宏昌 佐藤, 基佳
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AgriKnowledge

Immunity in Neonates of Mice Chronically Infected with *Babesia rodhaini*

Haruhisa SAKURAI, Hiroaki TAKAHASHI, Motoyoshi SATO[1)], Tsuneo HIROSE[1)], Atsushi SAITO, and Naoyoshi SUZUKI

Departments of Veterinary Physiology and [1)]Veterinary Clinical Radiology, Obihiro University, Obihiro, Hokkaido 080, Japan

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ABSTRACT. The resistance of newborn mice to infection with *Babesia rodhaini* was studied. No parasites were detected in the neonates or fetuses of both acutely infected and chronically infected mothers. However, the neonates born of chronically infected mice and nursed by normal uninfected mothers or by chronically infected mice were significantly more resistant to the infection than those from normal uninfected mice. In experimentally infected adult mice, the activities of macrophage migration inhibitory factor (MIF) in the supernate of concomitant cultivation of spleen cells with the lysate antigen of *Babesia* increased up to 6 weeks after infection. The degree of peritoneal macrophage phagocytosis in infants from chronically infected mothers was significantly greater than that of normal mice. The macrophage phagocytosis of parasitized erythrocytes was enhanced remarkably when the erythrocytes were exposed to immune serum just before addition to the macrophages.—KEY WORDS: babesiosis, lymphokines, mouse, neonatal immunity.

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In some protozoan infection, most of the neonates of chronically infected mother animals show a high degree of immunity to the homologous parasites as compared to those born of normal mothers [1, 10, 14, 16–19]. A foster experiment indicated that passive immunity was transferred primarily through the milk of the rodent [22]. Palmer [14] showed, however, that in rats infected with *Plasmodium berghei,* a degree of protection was transferred during pregnancy supporting the suggestion of Desowits [2] that neonates were sensitized by the soluble antigen of *P. berghei* which passed the placental barrier.

In our experimental studies on toxoplasmosis [18, 19], most of the neonates from immune mothers showed a significant resistance to *Toxoplasma* infection compared to these born of normal rats.

These phenomena demonstrated that T-lymphocytes had been sensitized transplacentally with a lymphokine to *Toxoplasma* before birth, and the lymphocytes returned to a non-sensitized state within several weeks after birth. Additionally, Omata *et al.* [11–13] suggested that the specific antigen-antibody complex could stimulate lymphocytes to induce proliferation and maturation of the peripheral immue system during the neonatal life. The goal of this study was to determine whether the protective immunity against *Babesia* infection was present in neonates of chronically infected mothers.

MATERIALS AND METHODS

Animals and infection: Laboratory bred conventional ICR/JCL, 6–8 weeks old female mice were used in this study. The Australian strain of *B. rodhaini* was obtained from the Kyushu Branch Laboratory of the National Institute of Animal Health. The parasite was maintained in the laboratory by passage at 4–5 day intervals. *B. rodhaini* parasitized erythrocytes (PE) were obtained from these mice and were

used to infect the experimental mice intraperitoneally (i.p.) at a dose of 1×10^2 PE per mouse. The severity of infection with *B. rodhaini* was assessed by the number of PE per 1,000 erythrocytes counted on a smear from tail blood stained with May-Crünwald and Giemsa.

Mice were treated with 4,4-diamino-dibenzamidine diaceturate (Ganaseg, E. R. Squibb and Sons Inc., Manila, Phillipines) administered intramuscularly (i.m.) at a dosage of 1.0 mg per mouse per day on 7, 8, 9, 11, 12 and 13 days postinoculation (p.i.). They were challenged with 1×10^4 PE per mouse i.p. 4 weeks after the first infection and the animals which survived 4 weeks after the challenge were used throughout the experiment as *Babesia* immune mice.

Babesia lysate antigen: *Babesia* lysate antigen (BLA) was prepared by the method described previously by Ishimine *et al.* [6] and Ito *et al.* [7]. *Babesia*-PE were washed 3 times with heparinized Hanks' balanced salt solution (HBSS, with 10 units heparin/m*l*) and once with Tc-199 by centrifugation at 750×G for 10 min at 4°C. The sediment was hemolyzed 5 or 6 times with warm 0.83% ammonium chloride solution, centrifuged at 45,000×G for 45 min at 20°C, washed 3 times with PBS, and centrifuged at 45,000×G for 45 min at 4°C. The sediment was resuspended in physiological saline. The suspension was frozen at −20°C and thawed at room temperature for 7 to 8 cycles, and then sonicated for 5 min at 100 W (Kubota Insonator, Model 200, Tokyo). The lysate product was centrifuged at 45,000×G for 45 min at 4°C. The supernate was filtered through a millipore membrane filter (0.3 μ, type HA, Millipore Co., Bedford, Mass, USA) and stored at −70°C until use. Total protein content of the BLA was estimated by the Lowry method [8] using bovine serum albumin fraction V as the standard.

Spleen cells and lymphokines: Cells were collected from the spleens of normal or infected mice. The cell separation was done by the Conray-Ficoll method [21]. The cells were washed twice with HBSS. After centrifugation at 450×G for 10 min, the resulting cells were resuspended in a medium (Tc-199) containing 10% heat inactivated calf serum (M+10%CS), and the concentration was adjusted to approximately 1×10^7 cells/m*l*. Immune and normal spleen cells were cultured with M+10%CS containing BLA (100 μg/m*l*) at 37°C for 48 hr in a humidified 5% CO_2 incubator. The supernate of immune spleen cell cultures, lymphokines (LKs), or normal spleen cell cultures were filtered through a membrane filter and store at −70°C until use.

Assay for macrophage migration inhibitory factor: The assay was performed by the modified agarose droplet method [5, 9, 15]. Guinea pigs were injected i.p. with 10 m*l* of 3% thioglycolate agar to stimulate peritoneal exudation. Three days later, peritoneal exudate cells (PEC) were harvested by washing the peritoneal cavity with 50 to 60 m*l* of HBSS. The cells obtained were wahsed 3 times with HBSS and resuspended in Tc-199 medium. The cell suspension was then mixed with agarose saline solution and maintained in a water bath at 37°C. One microliter of the PEC suspension was dropped into each well of the chamber (Lab Tek Product, USA) and cooled for 5 min at 4°C. Lymphokines (0.3 m*l*) were gently poured into each of two wells containing PEC agarose droplets. Wells containing M+10%CS served as controls. The chambers were incubated at 37°C for 24 hr in a humidified CO_2 incubator. The migration distance of macrophages was measured from the periphery of the droplet to that the radially migrated macrophages. The activity of macrophage migration inhibitory factor (MIF) of each of the LKs was calculated by the formula described by Sakurai *et al.* [18].

Phagocytosis test: Macrophage mono-

layers were prepared as described by Nagasawa *et al.* [9]. Peritoneal exudates containing a large number of macrophages were harvested by peritoneal washing with HBSS. The cells were centrifuged at 320×G for 5 min at 4°C. The sediment was suspended in M+10%CS and adjusted to a concentration of 1×10^6 mononucrear cells per m*l*. One milliliter of this suspension was placed in each well of a multidish tray (FB-15-24. Linbro Flow Lab., Inc., Conn., USA) containing a round coverslip, and incubated for 6 hr in a humidified 5% CO_2 incubator at 37°C. The cultures were washed 3 times with the same medium to remove non-adherent cells at 2 hr intervals. The cultures were reincubated with the same medium for 18 hr in a 5% CO_2 incubator and used as macrophage monolayers.

Babesia-PE were washed 3 times with HBSS and once with Tc-199 by centrifugation at 750×G for 10 min at 4°C. *Babesia* immune mouse fresh serum (BIFS), normal mouse fresh serum (NFS) or Tc-199 was added to the sediment at a volume ratio of 2:3. These mixtures were incubated at 37°C for 30 min in a water bath, and washed 3 times with HBSS and once with Tc-199. These treated erythrocytes were suspended in M+10%CS and added to the macrophage monolayers at a final concentration of 1×10^7 cells per well. The coverslips were taken out of the culture trays and stained with May-Grünwald and Giemsa at 30, 60 and 120 min after inoculation [6]. Macrophages with phagocytized PE were identified as "positive" macrophages and the rate was calculated from a total of 1,000 macrophages. Student's t-test was used to evaluate the significance of the results.

Vertical transmission of Babesia parasites from the acutely infected or immune mice to their fetuses: Parasite isolation was attempted by subinoculation of homogenized organs of fetuses or neonates i.p. into normal adult mice. The whole body of fetuses used at 2 weeks of gestation were homogenized. Only in the case of 3-week-old fetuses and neonates, the internal organs excluding gastrointestinal tract were homogenized. Mice at the end of the 1st week of pregnancy were infected with *Babesia* parasites. The fetuses were used for subinoculation 1 week after infection of the mother. The subinoculated mice were designated as group I. In group II, other pregnant mice were infected at the end of the 2nd week of pregnency and divided into 2 groups. The fetuses of one group were used for subinoculation at the end of the 3rd week of pregnancy. These subinoculated mice were designatd as group II-A. The newborn mice from the other group, as well as the *Babesia* immune mice were used for subinoculation, and were designated as group II-B and group III, respectively. The mortality rate of these subinoculated mice of each group was observed for 4 weeks.

Babesia infection of neonates of normal or Babesia immune mice: The *Babesia* immune female mice were challenged with *Babesia*-PE 1×10^2 per mouse. They were mated for 4 weeks following the challenge. Neonates and mothers were treated according to the method described by Palmer [14], but a slight modification was made as follows: (1) neonates born of normal mothers were nursed by normal mothers (N/n), and those born of immune mothers were nursed by immune mothers (I/i), (2) neonates born of immune mothers were put to normal mothers (I/n), and those born of normal mothers were put to immune mothers (N/i). The mothers of neonates were exchanged within 8 hr post partum. Each litter of neonates was divided into 2 groups. One group was inoculated with 1×10^2 *Babesia*-PE neonate within 1 week after birth and challenged with the similar number of PE 4 weeks after primary infection. These neonates were designated as group-A. The other

Table 1. The activity of macrophage migration inhibitory factor (MIF) in mice after primary infection and challenge with *B. rodhaini*

	Weeks after primary infection[a)]					
	0 (Normal)	1	2	3	4[b)]	6
MIF activity (%)	28[c)]	48	56	36	33	51

a) Infected with PE (1×10^2 per mouse).
b) Challenged with PE (1×10^4 per mouse) 4 weeks after primary infection.
c) $100\times\left(1-\frac{\text{Average distance of migration in the test material}}{\text{Average distance of migration in the control}}\right)$.

Table 2. MIF activity in infected mice[a)] after BLA[b)] injection

	Hours after BLA injection			
	3	6	12	24
MIF activity (%)	42[c)]	55	54	69

a) Infected 4 weeks before BLA injection (1×10^2 PE/mouse).
b) *Babesia* lysate antigen (100 μg of protein/mouse) injected intraperitoneally.
c) See Table 1.

half (group-B) was infected with 1×10^2 *Babesia*-PE per mouse 5 weeks after birth, at the time of the challenge in group-A. They were examined for mortality and parasitemia. Chi-square test was used to evaluate the results of mortality.

RESULTS

Appearance and degree of MIF activity: As shown in Table 1, MIF activity increased

Table 3. Phagocytic abilities of normal or *Babesia* immune mouse macrophages against parasitized erythrocytes treated with antibodies

Macrophages collected from	Incubation time (min)	Phagocytic percentage[a)] of macrophages against parasitized erythrocytes treated with		
		BIFS[b)]	NFS[c)]	Tc-199
Babesia immune mice	30	3.7±1.5	2.2±0.5	3.5±0.6
	60	14.6±4.4	6.4±1.7	10.1±1.4[d)]
	120	47.5±3.2[d)]	16.7±3.5	22.6±7.8
Normal mice	30	8.9±3.5	3.7±1.3	2.8±0.4
	60	8.8±3.0	5.0±2.8	4.2±1.8
	120	26.1±6.4	11.9±3.8	12.8±7.0

a) Treated erythrocytes were suspended in M+10%CS and added to the macrophage monolayers at a final concentration of 1×10^7 cells per well. The coverslips were stained at 30, 60 and 120 min after incubation. Macrophages with phagocytized erythrocytes were identified as "positive" macrophages for the phagocytosis and the rate of phagocytosis was calculated from 1,000 macrophages. Mean(percentage)±SE was calculated from the results of 3 independent experiments.
b) BIFS, *Babesia* immune mouse fresh serum.
c) NFS, normal mouse fresh serum.
d) Statistically significant difference compared to the case of normal mice macrophage at the same period ($p<0.05$).

Table 4. Examination for vertical transmission of *Babesia* parasites

Adult female mice	Number of fetuses or neonates	Parasitemia[a] (%)	Mortality of recipient mice (number dead/ number injected)
Acute infected[b]			
group I	4	0.20	0/ 2
	11	0.72	0/ 2
	16	0.14	0/ 2
group II-A	7	0.10	0/ 2
	13	0.51	0/ 2
	9	0.22	0/ 2
	6	2.40	0/ 2
group II-B	10	–[c]	0/10
	13	–[d]	0/13
	16	–[e]	0/16
Babesia immune			
group III	6	–	0/ 6
	7	–	0/ 7
	5	–	0/ 5

a) Number of parasitized erythrocytes per 100 erythrocytes in tail blood smears before sacrifice.
b) Group I. Normal mice in the 1st week of pregnancy infected with *Babesia* parasites: fetuses used for subincoulation 1 week after infection of the mother.
Group II-A. Normal mice infected at the end of the 2nd week of pregnancy; fetuses used for subinoculation at the end of the 3rd week of pregnancy.
Group II-B. Normal mice infected at the end of the 2nd week of pregnancy.
c~e) Died of babesiosis at the 7th, 2nd and 4th day after birth, respectively.

up to the 2nd week p.i. in *Babesia* infected mice. In the succeeding weeks, MIF activity tended to decrease during the 3rd and 4th weeks p.i. When the infected mice were challenged with *B. rodhaini* 4 weeks after primary infection (*Babesia* immune mice), MIF activity increased up to 51% 2 weeks after the challenge. Table 2 shows the MIF activity in the *Babesia* immune mice injected i.p. with BLA (100 μg/mouse). MIF activity was examined at 3, 6, 12 and 24 hr after injection. MIF activity increased up to the maximum of 69% at 24 hr.

Phagocytic ability of macrophages from normal and Babesia immune mice to the PE: The percentages of phagocytosis for the PE treated with Tc-199 medium (Tc-PE) in normal mouse macrophage monolayers at 30, 60 and 120 min incubations were 2.8, 4.2 and 12.8%, respectively (Table 3). However, in immune mouse macrophage monolayers, phagocytosis was 3.5, 10.1 and 22.6%. The phagocytosis of normal mouse macrophages incubated with normal-fresh-serum-treated PE (NFS-PE) at 30, 60 and 120 min after inoculation, was 3.7, 5.0 and 11.9%, respectively. In case of *Babesia*-immune-fresh-serum-treated PE (BIFS-PE), phagocytosis increased to 8.9, 8.8 and 26.1%. Phagocytosis of immune mouse macrophages incubated with NFS-PE at 30, 60 and 120 min after inoculation was 2.2, 6.4 and 16.7%, respectively. Phagocytic ability of immune macrophages was remark-

Table 5. Comparison of resistance against *Babesia* infection between infants from normal and *Babesia* immune mice

Group	Mortality of neonates after infection			
	Challenged (A)[a]		Not challenged (B)[b]	
	Number of dead/ number of infected	(%)	Number of dead/ number of infected	(%)
Infants born of immune mice,				
and nursed by immune mice (I/i)	6/35	17.1[c]	16/38	42.1[d]
and nursed by normal mice (I/n)	1/ 9	11.1[e]	8/16	50.0[f]
Infants born of normal mice,				
and nursed by immune mice (N/i)	20/33	60.6	30/35	85.7
and nursed by normal mice (N/n)	19/30	63.3	28/32	87.5

a) Neonates inoculated with *Babesia*-PE 1×10^2 per neonate within 1 week after birth and challenged in a similar fashion 4 weeks after primary infection.
b) Infants infected with *Babesia*-PE 1×10^2 per mouse 5 weeks after birth.
c~f) The statistically significant difference of mortality compared to N/i mice ($p<0.01$).

ably increased for BIFS-PE with respective percentages of 3.7, 14.6 and 47.5. Phagocytosis in immune mouse macrophages was greater than those in normal mouse macrophages. The percentage of phagocytized cells in immune mouse macrophages incubated with Tc-199 for 60 min was significantly greater than that in normal mouse macrophages (10.1 and 4.2%, $p<0.05$).

Vertical transmission and host resistance against Babesia infection: As shown in Table 4, 3 female mice and their 31 fetuses in group I, 4 female mice and 35 fetuses in group II-A, 3 female mice and 39 neonates in group II-B, and 3 female mice and 18 neonates in group III were examined in this experiment. None of the recipient mice, which were injected with homogenized material, died of babesiosis.

Immune resistance of the infants against *Babesia* infection was examined (Table 5). Mortality in group I/i-A and I/n-A was as low as 17.1 and 11.1%, respectively. On the other hand, 60.6 and 63.3% of the infants died in group N/i-A and N/n-A, respectively. Mortality in group N/i-B reached 85.7%. In contrast, mortality in group I/n-B and I/i-B reached 50.0% and 42.1%, respectively. It was noted that mortality in the four groups, I/i-B, N/i-A, I/n-B and N/n-A were close to each other (Table 5).

DISCUSSION

No vertical transmission of *Babesia rodhaini* was found in this experiment. However, the neonates from the immune mice both in group A (neonates infected with *Babesia*-PE 1 week after birth and challenged 4 weeks after primary infection) and group B (neonates infected with *Babesia*-PE 5 weeks after birth) showed a higher resistance against *Babesia* infection as compared with that from the normal mice. In our *in vitro* experiment, with the addition of BIFS-PE, the phagocytic ability of immune macrophages was higher as compared with phagocytosis of NFS-PE. This suggests that the serum antibody might play a part in the resistance against *Babesia* infection in neonates born of immune mice. It is accepted that a large proportion of protective antibodies is transferred from mother to infants mainly through the milk in the rodents [3, 16, 19, 22]. Furthermore, in rodents with *Plasmodium berghei*, the IgG antibodies in

the serum of neonates from immune mothers decreased gradually, with no serum IgG antibodies remaining at the 5th week after birth [10]. On the contrary, the infants born of normal and nursed by immune mothers showed no resistance against *Babesia* infection (N/i-A), while those born of immune and nursed by normal mothers showed remarkable protection (I/n-A). Furthermore, the immune infants fostered by normal mothers (I/n-B) also showed more resistance against single *Babesia* infection than mice in N/i-B. These findings suggest that the resistance of neonates born of immune mice is dependent on a factor, other than antibody, transferred during the fetal period. The antibody-parasite complex has possibly sensitized the immunocompetent cells of the neonates, and the challenge has triggered the secondary immune responses [11–13]. Desowits [2] suggested that a soluble antigen crosses the placental barrier and sensitizes the fetal immunocompetent cells in rats with *P. berghei*. In addition, Flynn *et al.* [4] and Teranishi [20] reported that the interleukin-1 (IL-1) was released from mononucrear phagocytes isolated from mouse and human placentae into culture medium and that its release was increased by stimulation of phagocytosis. These macrophages are stimulated by the soluble antigen opsonized by specific antibody.

The report that neonates of chronically infected mother rats showed a significant resistance to *Toxoplasma* infection compared to those born of normal rats [18] may support our hypothesis that macrophages and spleen cells may be possibly sensitized by soluble antigen or by lymphokines which have been transferred from the imuune mother. A possible explanation for this finding might be that fetal lymphocytes had been sensitized to *Babesia* before birth and that they returned to a non-sensitized state within several weeks after birth. Our experimental results, in which MIF activity increased in *Babesia* infected mice, may suggest that the cell-mediated immune response might play a large part in the resistance against *Babesia* infection. In consequence, the high resistance against *Babesia* immune mice might probably be caused by the same action of T-lymphocyte sensitization as in the neonates from *Toxoplasma* immune mice.

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要　約

バベシア感染マウス新生仔における *Babesia rodhaini* 感染に対する抵抗性：桜井治久・高橋宏昌・佐藤基佳[1]・広瀬恒夫[1]・斎藤篤志・鈴木直義（帯広畜産大学家畜生理学教室，[1]獣医臨床放射線学教室）——*Babesia rodhaini*（以下，バベシア）感染耐過マウスの脾臓細胞をバベシア抗原存在下で培養すると，培養上清（LKs）は，非感染マウス由来の脾臓細胞培養上清に比べて高い MIF 活性を示した。感染耐過マウスの腹腔マクロファージ（Mϕ）のバベシア貪食能は，非感染マウス Mϕ に比較して高進していた。バベシア感染急性期および感染耐過母マウスからの胎仔あるいは新生仔からは，原虫は分離されず，垂直感染は証明できなかった。感染耐過母マウスからの新生仔は，1週齢でバベシア感染に対して強い感染死防御能を示した。感染耐過母マウスからの新生仔は，非感染マウスで授乳しても，1～5週齢の攻撃に対して有意の感染抵抗性を示した。