Enzyme Activities Related to Glucose Metabolism in *Babesia microti* and *Babesia rodhaini*

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ABSTRACT. A comparative study was carried out on the glucose metabolism in *Babesia microti* (BM) and *Babesia rodhaini* (BR) by analyzing the enzyme activities. The lactate dehydrogenase (LDH) activity in BM showed significantly lower values than that in BR, whereas citrate synthase (CS) and malate dehydrogenase (MDH) activities were remarkably higher in BM. In addition, pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (KGDH), and succinate dehydrogenase (SDH) activities also tended to be higher in BM. Then, the change of enzyme activities related to the proliferation of parasites was examined. In BM infected mice, the parasitemia increased from day 15 to day 19 after inoculation (a.i.). While BM showed decrease of G6PD and LDH activities at day 19 a.i., it showed remarkably increased activities in CS and MDH (368 and 8,842 nmol/min·mg protein, respectively). In addition, PDH, ICDH, KGDH, and SDH activities also tended to increase from day 15 to 19 a.i. In BR infected mice, parasitemia increased from day 9 to day 12 a.i. LDH activity showed a considerable increase at day 12 a.i. (12,920 IU/mg·protein). Although CS and MDH activities also showed a slight increase at day 12 a.i., the activities of PDH, ICDH, KGDH and SDH didn't change from day 9 to 12 a.i. Since these changes observed in the enzyme activities of BM and BR seemed to be correlated with their proliferation, it was suggested that BM and BR depended on aerobic and anaerobic pathways, respectively, for their glucose metabolism.—KEY WORDS: *Babesia microti, Babesia rodhaini*, enzyme, glucose metabolism.

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Babesia microti (BM) and *Babesia rodhaini* (BR) are the major causative agents of babesiosis in mice. Although they were classified in the same genus, various differences have been observed between them. BM-infection was found to be non-lethal despite of the temporary hyperparasitemia, whereas BR-infection was always lethal in mice [6]. In addition, mice infected with the two *Babesia* species showed different immune responses [9, 10, 14, 18, 27]. Furthermore, some investigators reported that BM proliferated in lymphocytes, and suggested that it was questionable to classify this species in the genus of *Babesia* [29].

Investigations on the metabolic characteristics of protozoa had often been utilized for the classification [4, 5, 8, 11], development of drugs, and also the protectional measures against protozoan diseases. Moreover, protozoan metabolic characteristics were known to vary depending upon the stage of their life cycles and/or proliferation. As to *Babesia* species, Rickard [19] examined enzyme activities and intermediate products related to glucose metabolism in BR, and suggested that its major metabolic pathway was anaerobic glycolysis. In *Plasmodium knowlesi*, The increase in some of its glycolytic enzyme activities were proportional to the increase of parasitemia [21]. Additionally, in *Trypanosoma brucei* the glucose-metabolic pathways changed from an anaerobic to an aerobic type during its differentiation *in vitro* [7].

There are only a few reports on the comparative studies on the characteristics of glucose metabolism, or changes of the characteristics in the developmental stages of BM and BR during the course of infection. In this paper, comparative studies were carried out into the characteristics of glucose metabolism in BM and BR by enzymatic analysis.

MATERIALS AND METHODS

Animals: The ICR mice, female, 6–8 wks of age, were obtained from Charles River Laboratories (Atsugi, Japan).

Parasites: *Babesia microti* (Munich strain: BM) and *Babesia rodhaini* (Australian strain: BR) were maintained by weekly passages to mice in our laboratory.

Sample preparation: Parasitized erythrocytes (PRBC) were collected from BM- and BR-infected mice by cardiac puncture at approximately 10% parasitemia. The blood solution was adjusted with sterile saline to the concentration of 5×10^4 PRBC per m*l*. After 0.2 m*l* of PRBC solution per mouse was injected intraperitoneally to 30 mice for each species, blood was collected by cardiac puncture from the mice showing parasitemia of approximately 50–70%. Sample preparation was performed similarly as described by Rickard [19]. Briefly, blood collected into syringes, containing 0.1 m*l* of acid citrate dextrose (ACD solution) for each mouse, was pooled in a 15 m*l* tube and centrifuged at 1,600 g for 10 min at 4°C. Buffy coat was removed and resuspended in PBS (pH 7.4), followed by centrifugation. This process was repeated 3 times. Then the erythrocytes were lysed by 0.75% NH_4Cl in Tris-HCl solution (17 mM, pH 7.65) for 10 min on ice and centrifuged at 20,000 g for 20 min at 4°C, which was repeated 2 more times. Then, the obtained freed parasites were washed 3 times with PBS (pH 7.4, containing 5 mM ethylendiamine tetraacetic acid: EDTA) and centrifugation at 20,000 g for 20 min at 4°C, and the pellet was stocked at −80°C until use. Just before the

Table 1. Samples of *B. microti* and *B. rodhaini* at three different points after infection

	B. microti			*B. rodhaini*		
	M1	M2	M3	R1	R2	R3
Days after inoculation	15	17	19	9	11	12
Parasitemia (%)*	18.6	50.5	54.9	21.6	45.4	76.9#
Packed cell volume (%)*	44.1	32.8	22.2	44.7	40.6	22.1#

* Mean values of 10 mice for each sample.
\# Mean values of 20 mice.

enzyme assay, the samples were thawed at 4°C and mechanically ruptured by sonication with an ultrasonic disrupter (Tomy UD201, Tomy, Tokyo, Japan) at 80W for 15 min in ice. The supernatant was separated from cell debris by centrifugation at 20,000 g for 30 min at 4°C. Blood collected from 15 clinically healthy mice and processed in the same way served as controls.

For evaluation of enzyme activities during the course of infection, 40 mice were inoculated with BM. Parasitemia was monitored and the parasite samples were prepared as described above from 10 mice at each time at day 15, 17 and 19 after inoculation. These samples were referred to as M1, M2, and M3, respectively. For BR, 50 mice were inoculated and the samples were obtained form 10 mice at day 9 and 11, and from 20 mice at day 12 after inoculation. These samples were referred to as R1, R2, and R3, respectively (Table 1).

Enzyme assay: The enzyme activities were determined on 4 and 3 samples for BM and BR, respectively. For evaluation of the change of enzyme activities during the course of infection, values were determined only once on pooled samples.

The glucose-6-phosphate dehydrogenase [EC1.1.1.49] (G6PD) activity was determined by the method of Lohr and Waller [12]. Briefly, 1.0 m*l* of sample was mixed with 1.9 m*l* of 50 mM Tris-HCl buffer (pH 7.5, containing 0.4% EDTA) and 0.05 m*l* of 2.5% β-nicotineamide adenine dinucleotide phosphate (NADP; Sigma, St. Louis, MO, U.S.A.; containing 1% $NaHCO_3$), and-incubated at 25°C for 5 min. Then 0.05 m*l* of 1.3% D-glucose 6-phosphate (disodium salt; Sigma) was added and the increase of optical density at 340 nm was measured with a model 200–20 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) for 5 min. The lactate dehydrogenase [EC1.1.1.27] (LDH) activity was measured with a commercial kit (LDH Monotest, Boerhinger Mannheim, Mannheim, Germany) using the Video Chemistry System 600 (Nisshin Koki, Tokyo, Japan). The pyruvate dehydrogenase [EC1.2.4.1] (PDH) activity was determined by the method of Brown and Perham [3] with a slight modification. Briefly, 0.05 m*l* of sample solution was mixed with 1.8 m*l* of 82 mM potassium phosphate buffer (pH 7.5), 0.25 m*l* of 30 mM NAD, 0.2 m*l* of 2.25 mM CoA, 0.2 m*l* of 15 mM $MgCl_2$, 0.2 m*l* of 39 mM cystein and 0.1 m*l* of 6 mM thiamin pyrophosphate, and incubated at 37°C for 10 min. Then 0.2 m*l* of 30 mM pyruvate (Wako Pure Chemical, Osaka, Japan) was added and the increase of optical density at 340 nm was measured for 5 min. The malate dehydrogenase [EC1.1.1.37] (MDH) activity was determined by the method of Bergmeyer and Bernt [2]. Briefly, 2.5 m*l* of 0.1 M phosphate buffer (pH 7.4, containing 41.8 mM asparate) was mixed with 0.05 m*l* of 66 mM 2-oxoglutarate (Wako Pure Chemical), 0.05 m*l* of 12.1 mM β-nicotineamide adenine dinucleotide (reduced form: NADH; Sigma), and incubated at 20°C for 5 min. The decrease of optical density at 340 nm was measured for 5 min just after the addition of 0.1 m*l* of sample solution. The citrate synthase [EC4.1.3.7] (CS) activity was assayed by the method of Srere [28] with a slight modification. The 0.5 m*l* of sample solution was mixed with 2 m*l* of 0.1 mM Tris-HCl (pH 8.1), 0.2 m*l* of 10 mM 5,5'-dithiobis-(2-nitrobenzoate) (DTNB; dissolved in 0.1 mM Tris-HCl) and 0.02 m*l* of 5 mM acethylcoenzyme A (acethyl-CoA; Sigma), and incubated at 37°C for 5 min. Then 0.2 m*l* of 25 mM oxalacetate (Sigma; neutralized with KOH) was added and the increase of optical density at 412 nm was measured for 5 min. The α-ketoglutarate dehydrogenase [EC1.2.4.2] (KGDH) activity was measured by the method of Schoonen *et al.* [23] with a slight modification. The 0.3 m*l* of the sample solution was mixed with 1.5 m*l* of 0.11 M potassium phosphate buffer (pH 7.5), 0.3 m*l* of 30 mM NAD (Sigma), 0.1 m*l* of 2.25 mM CoA, 0.2 m*l* of 12 mM $MgCl_2$ and 0.2 m*l* of 39 mM cysteine (Wako Pure Chemical), and incubated at 37°C for 10 min. Then 0.2 m*l* of 30 mM pyruvate (Wako Pure Chemical) was added and the increase of optical density at 340 nm was measured for 5 min. The succinate dehydrogenase [EC1.3.99.1] (SDH) activity was assayed by the method of Ackrell *et al.* [1] with a slight modification. Briefly, 0.2 m*l* of 20 mM Tris-HCl buffer (pH 7.35, containing 100 μM EDTA) was mixed with 0.25 m*l* of 10 mM potassium ferricyanide (Wako Pure Chemical), and 0.3 m*l* of 200 mM sodium succinate (Wako Pure Chemical), and incubated at 37°C for 10 min. The reaction was started by adding 0.25 m*l* of sample solution and the optical density at 420 nm was measured for 5 min. The isocitrate dehydrogenase [EC1.1.1.] (ICDH) activity was determined by the method of Plaut [17] with a slight modification. The 2.0 m*l* of 33.3 mM Tris-acetate buffer (pH 7.20) was mixed with 0.2 m*l* of 0.66 mM adenine dinucleotide phosphate (ADP; Sigma), 0.05 m*l* of 0.3 mM NAD, 0.2 m*l* of 5.3 mM *threo*-DsLs-isocitrate (Sigma) and 0.2 m*l* of 1.3 mM $MnCl_2$, and incubated at 37°C for 10 min. Then 0.35 m*l* of sample solution was added and the increase of optical density at 340 nm was measured for 5 min.

Statistical analysis: Data were analyzed for significance by Student's *t* test.

RESULTS

Enzyme activities: Enzyme activities in BM and BR are

Table 2. Enzyme activities in *B. microti* and *B. rodhaini*

Enzyme	Activity (n mol/min·mg protein)	
	B. microti (n=4)[a)]	*B. rodhaini* (n=3)[b)]
G6PD	14±7.5	20.3±2.1
LDH[c)]	2110±375#	6110±2840#
PDH	0.9±0.7	ND
CS	133±111	20±10
ICDH	3.5±2.4	1.2±0.6
KGDH	0.7±0.9	ND
SDH	8.6±2.4	7.4±9.2
MDH	1330±850	439±56

a, b) Values are mean±SD determined on 4 and 3 samples for *B. microti* and *B. rodhaini*, respectively, each of which was prepared on different occasions from 30 mice and stocked until use as described in Materials and Methods.
c) IU/mg protein, #: Significant difference ($p<0.05$) between *B. microti* and *B. rodhaini*, ND: Not detected.

shown in Table 2. G6PD activities of BM were similar to those of BR (BM; 14±7.5, BR; 20.3±2.1 nmol/min·mg protein). LDH activity in BM (2,110±375 IU/mg protein) was significantly ($p<0.05$) lower than that in BR (6,110±2,840 IU/mg protein), whereas CS and MDH activities were higher in BM compared with BR. In addition, the activities of PDH, ICDH, and KGDH in BM tended to be higher than those in BR.

Changes of enzyme activities during the course of infection: In BM infected mice, G6PD and LDH activities decreased during the course of infection, whereas CS and MDH activities increased (CS: from 15.1 to 368 nmol/min·mg protein, MDH: from 393 to 8,840 nmol/min·mg protein) which was remarkable in M3 (Fig. 1, Table 3). PDH, ICDH, KGDH, and SDH activities also showed an increase in M3. In BR infected mice, G6PD activities showed a decrease in R3. LDH activity remarkably increased from R2 to R3 (5,200 and 12,900 IU/mg protein, respectively). CS and MDH activities also showed a slight increase in R3, whereas no change was observed in PDH, ICDH, KGDH, and SDH activities (Fig. 1, Table 3). Comparing M3 and R3, which showed the remarkable changes in several enzyme activities as described, BM showed significantly lower LDH activity than BR. On the contrary, BM exhibited nearly 8 and 11 times higher activities than BR in CS and MDH, respectively. In addition, PDH, ICDH, KGDH, and SDH activities were also higher in BM (Table 3).

Table 3. Enzyme activities in *B. microti* and *B. rodhaini* at three different points in the course of infection

Sample	Activity (n mol/min·mg protein)[a)]							
	G6PD	LDH[b)]	PDH	CS	ICDH	KGDH	SDH	MDH
M1	21.5	4320	0.8	15.1	ND	1.2	6.6	393
M2	14.3	2930	1.9	71.0	3.2	ND	16.0	663
M3	12.3	1630	1.8	368.0	7.2	2.1	2ND	8840
R1	37.3	6310	1.3	15.4	1.3	ND	6.6	442
R2	31.5	5200	ND	15.2	ND	ND	0.8	393
R3	10.4	12900	ND	44.1	1.6	ND	6.6	786

a) Enzyme activities were determined once on the pooled samples.
b) IU/mg protein.
ND: Not detected

DISCUSSION

In *Plasmodium* species, glycolysis to lactate was the major energy-producing pathway in the erythrocytic stages. However, some of the *Plasmodium* species were reported to possess full tricarboxylic acid (TCA) cycle activity [26]. Another protozoan, *Eimeria tenella*, was reported to utilize the mannitol cycle for glycolysis like fungi [22]. In *Babesia* species, Momen *et al.* [16] reported that BM showed a low LDH activity. However, Mackenzie *et al.* [13] demonstrated that BM produced lactate as a sole metabolite when analyzed by nuclear magnetic resonance (NMR). Rickard [20] reported that BR-infected erythrocytes showed no greater ratio of oxygen uptake for glucose utilization than that found in normal erythrocytes, and suggested that the major glucose metabolic pathway in BR was anaerobic glycolysis. In the present study, BM showed significantly lower LDH, and higher CS and MDH activities than those determined in BR. The ratio of TCA cycle enzyme activities to glycolytic enzyme activities (MDH/LDH was 0.63 in BM and 0.07 in BR, respectively) was extremely higher in BM than that of BR. In addition, BM showed higher activities also in CS, PDH, ICDH, KGDH, and SDH, as well as MDH, than those in BR. Thus, these differences in the activities of BM and BR were considered to reflect their dependence on aerobic and anaerobic pathways in glucose metabolism, respectively.

On the other hand, Sander *et al.* [21] described the glucose metabolites in erythrocytes infected with *P. knowlesi* and suggested that the parasite utilized an anaerobic glycolysis. They also found that the increase of activities in some of the glycolytic enzymes, such as hexokinase, aldolase and enolase, could be correlated with the increase of parasitemia. Meier *et al.* [15] also reported a stage specific expression of aldolase isoenzymes in *P. berghei* during its development from sporozoits to merozoits. Another protozoan parasite, *T. brucei*, was reported to alter rapidly its TCA cycle enzyme activities during *in vitro* differentiation of bloodstream form to procyclic stages [7]. These reports suggested the possibility that *Babesia* also change their metabolic characteristics in the course of infection.

In BM infected mice, a decrease in LDH activity was associated with the increase of parasitemia. Contrary to this decrease of LDH activity, CS, PDH, ICDH, KGDH, and MDH activities increased in M3, whereas in BR infected mice, LDH activity remarkably increased in R3

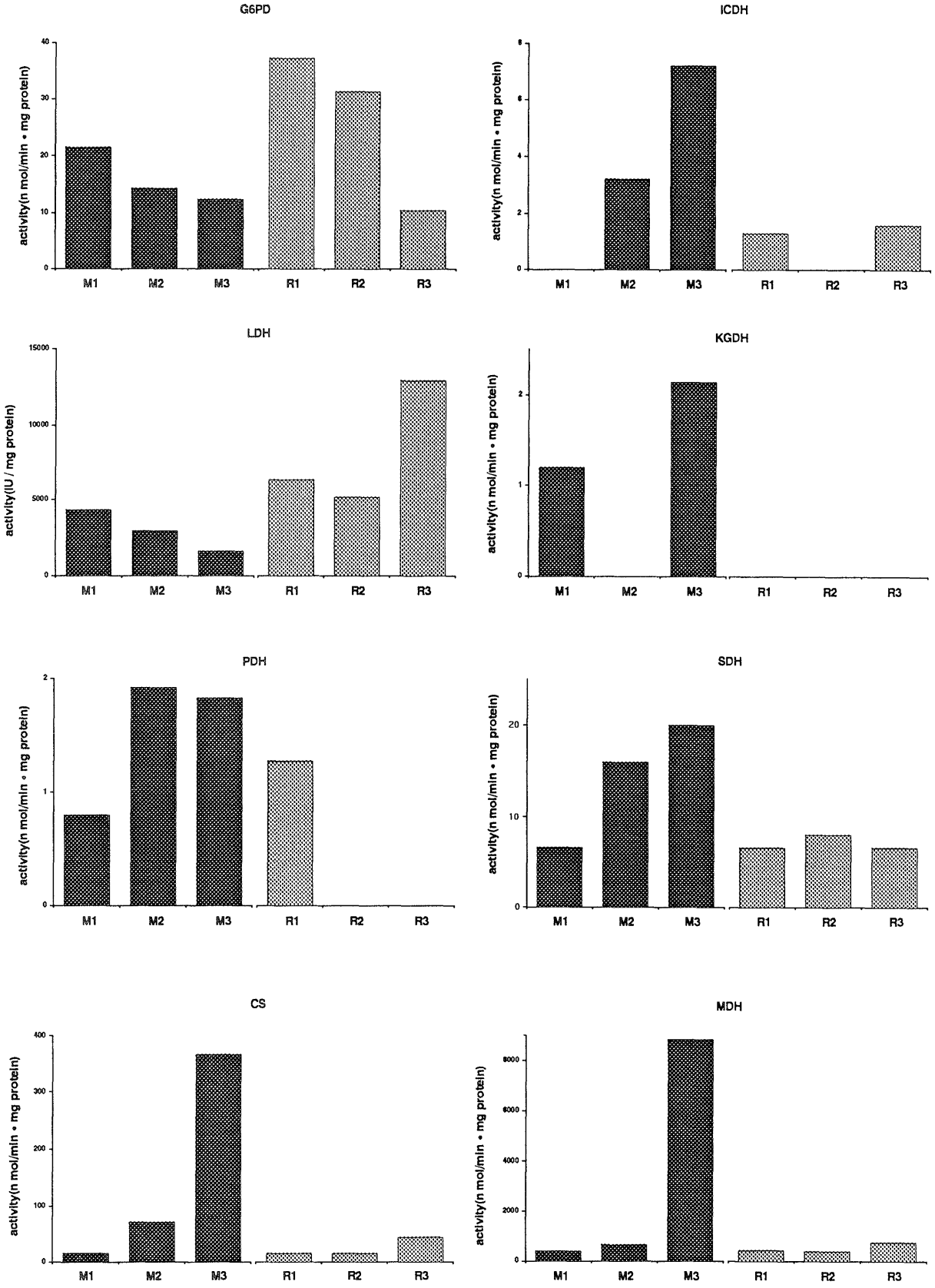

Fig. 1. Changes of the enzyme activities of *B. microti* and *B. rodhaini* in the course of infection. The values were determined once on the pooled samples.

with hyperparasitemia. A high parasitemia did not always reflect the proliferation of parasites in host cells, however, it was generally accepted as a result of proliferation of parasites. Thus, changes in enzyme activities in BM were considered to be correlated with proliferation. In *Plasmodium* species, the substantial activity of TCA cycle has been demonstrated only in *P. lophurae* and *P. gallinaceum* [24–27], the parasites which infect avian species where the host erythrocytes have full TCA cycle activity. Therefore, considerably high activities of TCA cycle enzymes observed in BM might have supported the possibility that this species has a schizogony stage in lymphocytes, as described by Young and Morzaria [30].

From these results, it seemed that BM and BR utilized mainly aerobic and anaerobic pathways, respectively, for their glucose metabolism.

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