Use of canine red blood cell with high concentrations of potassium, reduced glutathione, and free amino acid as host cells for in vitro cultivation of Babesia gibsoni

Masahiro Yamasaki, DVM; Hiroyuki Asano, DVM; Yayoi Otsuka, DVM; Osamu Yamato, DVM, PhD; Motoshi Tajima, DVM, PhD; Yoshimitsu Maede, DVM, PhD

Objective—To determine the usefulness of canine RBC with high concentrations of potassium, reduced glutathione (GSH), and amino acids (HK cells) for in vitro cultivation of Babesia gibsoni.

Animals—RBC were obtained from 3 dogs that had inherited HK cells and from 3 genetically unaffected dogs that, therefore, had RBC with lower potassium (LK) concentrations (ie, LK cells).

Procedures—First, B gibsoni were cultivated using HK or LK cells in alpha-modification of Eagle medium, consisting of Earle salts with glutamine and without ribosides, deoxyribosides, and sodium bicarbonate under a humidified atmosphere containing 5% CO₂ at 37 C. Second, parasites were cultivated with LK- or HK-cell lysates. Finally, HK cells were separated into 3 fractions (bottom, middle, top layers) by density gradient centrifugation, and B gibsoni were cultivated with each of the HK-cell fractions. In addition, the concentrations of free amino acids and reduced glutathione (GSH) in each HK-cell fraction were measured.

Results—B gibsoni preferentially multiplied in HK-cell cultures rather than in LK-cell cultures. Furthermore, the addition of HK-cell lysate to the culture medium resulted in enhanced multiplication of the parasites. Higher multiplication of the parasites was observed in HK cells from the top layer, compared with HK cells from the middle and bottom layers. The HK cells from the top layer had higher concentrations of glutamate, aspartate, and GSH, compared with HK cells from the middle and bottom layer.

Conclusions—Canine HK cells are useful host cells for in vitro cultivation of B gibsoni, and the high concentrations of glutamate, aspartate, and GSH may result in enhancement of multiplication of the parasites in HK cells. (Am J Vet Res 2000;61:1520–1524)

Babesia gibsoni is a known blood protozoan in dogs and a causative pathogen of hemolytic anemia in infected dogs. To our knowledge, the life cycle of the parasite and pathogenesis of the anemia, however, have not been elucidated. To clarify these issues, in vitro culture of the parasites is needed. Successful in vitro culture of B gibsoni was reported by Murase et al.1 In their study, B gibsoni multiplied better in reticulocytes than in mature RBC.2 Reticulocytes are immature RBC and differ from mature RBC. Cytoplasm of reticulocytes contain ribosomes and mitochondria, and reticulocytes have higher concentrations of ATP, reduced glutathione (GSH), free amino acids, and nucleic acids than the mature RBC.3 Furthermore, canine reticulocytes possess Na-K-ATPase activity but lose it rapidly during maturation into RBC.4 These biochemical and morphologic characteristics of reticulocytes may affect the multiplication of parasites, whereas the cause of the predilection of B gibsoni for reticulocytes remains to be determined.

As we reported previously, some dogs have RBC containing high concentrations of potassium (HK), GSH, and free amino acids as a result of an inherited high Na-K-ATPase activity (ie, HK cells). These characteristics of HK cells are similar to those of reticulocytes. It was expected, therefore, that B gibsoni may multiply in HK cells and that HK cells may be useful for the cultivation of B gibsoni instead of reticulocytes, as reticulocytes are not easily prepared. The purpose of the study presented here was to investigate the usefulness of HK cells for the in vitro cultivation of B gibsoni.

Materials and Methods

Preparation of RBC—Red blood cells were obtained from 3 dogs that had inherited HK cells and from 3 genetically unaffected dogs that, therefore, had RBC with lower potassium (LK) concentrations (ie, LK cells). Dogs with HK cells have been maintained since 1986 in our laboratory. Dogs had body weights of 10 to 15 kg. Blood samples were supplemented with heparin and centrifuged at 900 X g for 5 minutes at room temperature (approx 25 C). After removal of the plasma and buffy coat, packed RBC were resuspended in dog plasma to yield a PCV of 50% and filtered through an alpha-cellulose/microcrystalline cellulose column to remove leukocytes and platelets.5 Filtered cells were washed 3 times with 10 mM PBS solution (pH 7.4).

Fractionation of HK cells—The HK cells were fractionated by use of Percoll discontinuous density gradient centrifugation. Washed HK cells were resuspended in PBS solution to yield a PCV of 30% and fractionated by density gradient centrifugation. Percoll® discontinuous density gradient solutions were prepared containing 45, 60, and 67.5% (vol/vol) Percoll in 150 mM NaCl, 0.1% (wt/vol) bovine serum albumin, and 20 mM Hepes/Tris (pH 7.5). Specific gravities of the solutions were 1.070, 1.090, and 1.100 g/ml.
respectively. The HK cell suspension was layered over the Percoll gradient solution and centrifuged at 800 X g for 15 minutes at room temperature (approx 25 C). After centrifugation, the cells distributed in the interfaces of the 3 Percoll solutions and the cells with a specific density of < 1.100 g/ml were collected and designated as top, middle, and bottom layer cells. Each cell fraction was washed twice with PBS solution.

Preparation and culture of gibsoni—For preparation of B gibsoni-infected RBC, blood was collected from 3 experimental B gibsoni-infected dogs with parasitemia rates of 3.9 to 8.2% and washed with PBS solution. Babesia gibsoni was cultivated on the basis of the method of Murase et al1 with some modifications. Washed cells were resuspended in a culture medium consisting of 60% alpha-modification of Eagle medium (alpha-MEM), consisting of Earle salts with glutamine and ribosides, deoxyribosides, and sodium bicarbonate, supplemented with sodium pyruvate (0.11 mg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% serum from clinically normal dogs to yield a PCV of 3%. For cultivation of the parasites, the B gibsoni-infected RBC were mixed with 9 volumes of each cell suspension, and then 100 µl of a given mixed suspension was placed in each well of a 96-well flat-bottomed microculture plate and incubated at 37 C under a humidified atmosphere containing 5% CO2 for 6 days. Every 24 hour, 60 µl of the culture supernatant was removed and replaced with an equal volume of fresh culture medium. One well was used every day to make a thin smear sample. Percentage of parasitemia was calculated by counting the number of parasitized cells per 2,000 cells.

For the culture of B gibsoni with lysate, washed HK and LK cells were resuspended in the alpha-MEM to yield a PCV of 50% and lysed by 3 cycles of freezing in liquid nitrogen and thawing at room temperature (approx 25 C). After centrifugation at 15,000 X g for 30 minutes at 4 C, the supernatant was collected and filtered using a 0.45-µm pore-size filter. Respective filtered supernatants were used as HK- and LK-cell lysates. Lysate medium consisted of 50% HK- or LK-cell lysates, 10% alpha-MEM, and 40% serum from clinically normal dogs. Babesia gibsoni was cultivated in each lysate medium.

RBC indices and intracellular concentrations—The RBC indices of each cell type were examined, using an automatic blood cell counter.1 Concentrations of GSH in each cell type were measured according to the method of Beutler.7 To measure the intracellular concentrations of free amino acids, each cell suspension was resuspended in 154 mM NaCl to yield a PCV of 50% and lysed by freezing and thawing. Then, equal volumes of lysate and 5% sulfosalicylic acid were mixed and centrifuged at 8,000 X g for 10 minutes. Supernatants were stored at –80 C until use as samples for the free amino acid analysis. Free amino acids were analyzed, using an automatic analyzer.

Statistical analysis—Statistical analysis was performed using a Student t-test. Unless otherwise indicated, data of HK cells were compared to those of LK cells. Also, data from each of the 3 fractions of HK cells were tested. The difference between data was considered to be significant if the P value was < 0.05.

Results

Multiplication of B gibsoni in HK and LK cells—

When B gibsoni-infected RBC were cultivated with either HK cells or LK cells, the numbers of parasitized cells began to increase on day 1. By day 6, a substantial difference in the multiplication of the parasite was observed between HK- and LK-cell cultures. In HK-cell cultures, the amount of parasitemia increased parabolically and reached 4.5 ± 0.9% at day 6, whereas the highest parasitemia attained in LK-cell culture was 2.1 ± 0.7% at day 2 and gradually decreased thereafter (Fig 1).

Effect of lysates of HK and LK cells on the multiplication of B gibsoni—To help determine the cause of the difference between the multiplication of B gibsoni in HK- and LK-cell cultures, parasites were cultivated with LK cells and HK- or LK-cell lysate. The amount of

![Figure 1](https://example.com/fig1.png)

Figure 1—Percentage of parasitized cells when Babesia gibsoni was cultivated with canine RBC with high potassium (HK; squares) or low potassium (LK; circles) concentrations. Data are expressed as mean ± SD (n = 3). *Significantly (P < 0.05) different from the value for the LK-cell culture.

![Figure 2](https://example.com/fig2.png)

Figure 2—Percentage of parasitized cells when Babesia gibsoni were cultivated with canine LK cells and HK- (closed squares) or LK- (closed circles) cell lysate. LK-cell culture (open circles) is shown as a control. Data are expressed as mean ± SD (n = 3). *Significantly (P < 0.05) different from control values.
parasitemia in HK-cell lysate culture was significantly higher than that in the culture without lysate (control culture), whereas the multiplication of the parasites was not enhanced in LK-cell lysate culture, compared with control culture conditions (Fig 2).

Multiplication of *B. gibsoni* in fractionated HK cells—HK cells were separated into 3 fractions, clearly distinguished by differences in mean cell volume (MCV) and mean corpuscular hemoglobin concentration (MCHC), by Percoll discontinuous density gradient centrifugation, whereas LK cells were distributed only in the bottom layer (Table 1, Fig 3). When *B. gibsoni* was cultivated with each of the HK-cell fractions, the amount of parasitemia was significantly higher in the top-layer cell culture than in the bottom-layer cell culture. The parasitemia in the middle-layer cell culture was intermediate between those in the top- and bottom-layer cell cultures (Fig 4).

Concentrations of free amino acids and GSH in HK and LK cells—The HK cells had significantly higher concentrations of aspartate, glutamate, glutamine, histidine, proline, and GSH than LK cells (Table 2). Among the HK-cell fractions, top-layer cells possessed the highest concentrations of aspartate, glutamate and GSH, and bottom-layer cells had the lowest concentra-

Table 1—Indices of canine RBC with low potassium (LK cells) and high potassium (HK cells) concentrations and of fractionated HK cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LK cells</td>
<td>62.8 ± 2.20</td>
<td>20.5 ± 1.26</td>
<td>32.6 ± 0.89</td>
</tr>
<tr>
<td>HK cells</td>
<td>77.8 ± 5.18†</td>
<td>21.1 ± 1.86</td>
<td>27.0 ± 1.24†</td>
</tr>
<tr>
<td>Top layer</td>
<td>85.7 ± 3.76*</td>
<td>21.3 ± 1.56</td>
<td>24.8 ± 1.01*</td>
</tr>
<tr>
<td>Middle layer</td>
<td>73.3 ± 4.08*</td>
<td>22.0 ± 1.44</td>
<td>30.0 ± 0.78*</td>
</tr>
<tr>
<td>Bottom layer</td>
<td>64.5 ± 2.61</td>
<td>22.5 ± 0.99</td>
<td>34.8 ± 0.12</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (n = 3).

†Significantly (P < 0.05) different from value for bottom-layer cells.

*Significantly (P < 0.05) different from value for LK cells.

MCV = Mean corpuscular volume. MCH = Mean corpuscular hemoglobin. MCHC = Mean corpuscular hemoglobin concentration.

Table 2—Concentrations of reduced glutathione (GSH) and free amino acids in canine LK and HK cells

<table>
<thead>
<tr>
<th>Substance</th>
<th>LK cells (nmol/10^7 RBC)</th>
<th>HK cells (nmol/10^7 RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.21 ± 0.010</td>
<td>0.43 ± 0.197</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.15 ± 0.009</td>
<td>0.39 ± 0.200</td>
</tr>
<tr>
<td>Aspartagine</td>
<td>0.03 ± 0.008</td>
<td>0.11 ± 0.053</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.06 ± 0.002</td>
<td>2.63 ± 1.248*</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.07 ± 0.002</td>
<td>7.24 ± 3.512*</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.19 ± 0.008</td>
<td>6.78 ± 3.030*</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.11 ± 0.012</td>
<td>0.33 ± 0.155</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.05 ± 0.001</td>
<td>0.23 ± 0.113*</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.04 ± 0.002</td>
<td>0.06 ± 0.027</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.06 ± 0.004</td>
<td>0.06 ± 0.025</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.16 ± 0.010</td>
<td>0.45 ± 0.220</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.04 ± 0.003</td>
<td>0.09 ± 0.044</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.04 ± 0.002</td>
<td>0.06 ± 0.042</td>
</tr>
<tr>
<td>Proline</td>
<td>0.09 ± 0.005</td>
<td>0.34 ± 0.132*</td>
</tr>
<tr>
<td>Serine</td>
<td>0.11 ± 0.012</td>
<td>0.21 ± 0.098</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.18 ± 0.014</td>
<td>0.22 ± 0.096</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.12 ± 0.001</td>
<td>0.19 ± 0.087</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.06 ± 0.005</td>
<td>0.15 ± 0.055</td>
</tr>
<tr>
<td>Valine</td>
<td>0.08 ± 0.003</td>
<td>0.14 ± 0.068</td>
</tr>
<tr>
<td>GSH</td>
<td>0.72 ± 0.374</td>
<td>3.83 ± 0.482*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of 3 experiments (n = 3).

*Values are significantly (P < 0.05) higher than those of LK cells.
amounts in HK cells than in LK cells, seemed to play and proline, which were in significantly higher and GSH in HK cells. In contrast, glutamine, histidine, and GSH among the fractionated HK cells. These results indicate that the high amount of multi-
parasites was observed in the top-layer cell culture. Because HK cells contain high concentra-
tions of glutamate, aspartate, glutamine, histidine, and parasites. These results suggest that HK cells may be responsible for the multiplication of the parasites. These results indicate that reactive oxygen generated through a sodium-dependent transport system for glutamate and aspartate. Interestingly, glutamate and aspartate transport were greatly accelerated in the canine HK cells by the function of Na-K-ATPase, which is not detected in LK cells, resulting in high accumulation of glutamate and aspartate in HK cells. It thus appeared that B gibsoni may also use glutamate for its growth in RBC. In addition, aspartate transcarbamylase, which is an enzyme of the pathway of de novo pyrimidine biosynthesis, was detected in B rohdaini, suggesting that aspartate was used to synthesize the nucleic acids for the parasites. Thus, aspartate also may play an important role in the multiplication of B gibsoni.

Table 3—Concentrations of reduced glutathione (GSH) and free amino acids in fractionated canine HK cells

<table>
<thead>
<tr>
<th>Substance</th>
<th>Top-layer cells (nmol/10^6 RBC)</th>
<th>Middle-layer cells (nmol/10^6 RBC)</th>
<th>Bottom-layer cells (nmol/10^6 RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.22 ± 0.033</td>
<td>0.21 ± 0.026</td>
<td>0.21 ± 0.036</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.33 ± 0.124</td>
<td>0.30 ± 0.055</td>
<td>0.31 ± 0.098</td>
</tr>
<tr>
<td>Asparagine</td>
<td>TA</td>
<td>TA</td>
<td>TA</td>
</tr>
<tr>
<td>Aspartate</td>
<td>4.50 ± 1.367</td>
<td>3.54 ± 0.195*</td>
<td>2.37 ± 0.409</td>
</tr>
<tr>
<td>Glutamate</td>
<td>22.7 ± 6.312*</td>
<td>13.3 ± 0.852*</td>
<td>6.51 ± 1.954</td>
</tr>
<tr>
<td>Glutamine</td>
<td>6.12 ± 1.796</td>
<td>5.72 ± 0.553</td>
<td>6.63 ± 1.233</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.21 ± 0.078</td>
<td>0.19 ± 0.050</td>
<td>0.18 ± 0.064</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.34 ± 0.198</td>
<td>0.28 ± 0.072</td>
<td>0.26 ± 0.081</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.05 ± 0.016</td>
<td>0.04 ± 0.012</td>
<td>0.05 ± 0.016</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.06 ± 0.027</td>
<td>0.06 ± 0.025</td>
<td>0.07 ± 0.034</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.70 ± 0.343</td>
<td>0.58 ± 0.212</td>
<td>0.57 ± 0.281</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.08 ± 0.023</td>
<td>0.08 ± 0.004</td>
<td>0.08 ± 0.012</td>
</tr>
<tr>
<td>Phenylylalanine</td>
<td>0.04 ± 0.008</td>
<td>0.04 ± 0.003</td>
<td>0.04 ± 0.008</td>
</tr>
<tr>
<td>Proline</td>
<td>0.11 ± 0.030</td>
<td>0.11 ± 0.024</td>
<td>0.10 ± 0.056</td>
</tr>
<tr>
<td>Serine</td>
<td>0.20 ± 0.081</td>
<td>0.17 ± 0.011</td>
<td>0.19 ± 0.025</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.05 ± 0.012*</td>
<td>0.07 ± 0.034</td>
<td>0.11 ± 0.030</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.19 ± 0.057</td>
<td>0.17 ± 0.011</td>
<td>0.19 ± 0.025</td>
</tr>
<tr>
<td>Valine</td>
<td>TA</td>
<td>TA</td>
<td>TA</td>
</tr>
<tr>
<td>GSH</td>
<td>6.28 ± 1.052*</td>
<td>5.15 ± 0.870</td>
<td>3.35 ± 0.398</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of 3 experiments (n = 3). Values are significantly different from for bottom cell (P < 0.05). *Trace amount (< 0.01 nmol/10^6 RBC) of free amino acids.

In RBC, however, GSH has been thought to participate in intracellular redox reactions and to protect hemoglobin or membrane proteins from oxidative stress. Results of our previous study indicate that GSH is indispensable for RBC defense against the oxidative damage induced by acetylphenylhydrazine (APH; a strong oxidant drug), in sheep RBC, which have an inherited deficiency of GSH. It is known that the growth of malaria parasites in glucose 6-phosphate dehydrogenase (G6PD)-deficient RBC, which had a low GSH concentration as the result of the enzyme deficiency, is more readily inhibited than the growth in normal cells when oxidative stress is generated in the cells. We have previously reported that oxidative damage to RBC was induced by the multiplication of B gibsoni, and suggested that reactive oxygen species may be generated by the parasites during cultivation. These results indicate that reactive oxygen generated by the multiplication of the parasites may injure not only host RBC but also the parasites themselves, and the parasites may need a defense system, such as GSH, against oxidative damage for their intracellular multi-
plication. Because canine HK cells had increased pro-
tection against oxidative damage induced by APH, compared with LK cells with normal GSH content, the high concentration of intracellular GSH may be an important factor in promoting the high amount of mul-
tiplication of B gibsoni in HK cells.

In conclusion, canine HK cells are useful host cells for in vitro cultivation of B gibsoni. Furthermore, the high concentrations of glutamate, aspartate, and GSH in HK cells may result in the high amount of multiplication of B gibsoni in the cells. In addition, dogs posses-
sing HK cells, which appear to be distributed throughout Japan, Korea, and America, may be more susceptible to infection with Babesia parasites than clinically normal dogs.

It is known that several Babesia species, B bovis, B bigemina, and B microti, possess a glutamate dehydro-
genase that is capable of oxidizing glutamate to α-ketoglutarate, indicating that the further metabolism of α-ketoglutarate via the Krebs cycle could provide an energy source for those parasites in RBC. Because most mammalian RBC are impermeable to glutamate, intracellular glutamate is thought to be transformed from glutamine, which can penetrate the cells. In contrast, canine and feline RBC are permeable to glutamate through a sodium-dependent transport system for glutamate and aspartate. Interestingly, glutamate and aspartate transport were greatly accelerated in the canine HK cells by the function of Na-K-ATPase, which is not detected in LK cells, resulting in high accumulation of glutamate and aspartate in HK cells. It thus appeared that B gibsoni may also use glutamate for its growth in RBC. In addition, aspartate transcarbamylase, which is an enzyme of the pathway of de novo pyrimidine biosynthesis, was detected in B rohdaini, suggesting that aspartate was used to synthesize the nucleic acids for the parasites. Thus, aspartate also may play an important role in the multiplication of B gibsoni.

Discussion

Results of our study indicate that B gibsoni prefer-
entially multiplied in HK cells rather than in LK cells. Furthermore, the addition of HK-cell lysate to the LK-
cells was significantly less that that in bottom-
layer cells, whereas it was almost the same in HK and LK cells.
References


