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In Vitro Cultivation and Infectivity of *Babesia gibsoni*

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Abstract

Basic conditions for *in vitro* cultivation of *Babesia gibsoni* were examined. The parasites multiplied in canine red blood cells in RPMI-1640 medium (pH 7.3) supplemented with 20% canine serum under a gaseous environment of 5% CO_2 at 37°C. Higher parasitemia was obtained by exchanging the medium at 24-h interval. Under the optimum conditions determined in the present study, the percentage of parasitized red blood cells after incubation for 7 days increased about 9 times more than that at the initiation of the culture. The parasites were maintained for at least 28 days by exchanging a part of the red blood cells on every 7th day after incubation. The long-term cultured parasites, which were in dot form in red blood cells, had a potency to cause severe anemia in dogs.

Key words: *Babesia gibsoni, In vitro* cultivation, Infectivity, Hemoprotozoa, Canine babesiosis

Introduction

Babesia gibsoni is one of tick-borne hemoprotozoan parasites in dogs. The parasites cause severe anemia in the animals, although the parasitemia is lower than those in other babesioses of animals (Mahoney and Goodger, 1981). *In vitro* cultivation of *B. gibsoni* has been reported by Murase *et al.* (1991). However, the cultivation period was about 15 days and the percentage of parasitized red blood cells was 4.0% at the maximum. They also have not reported the variables of the cultural conditions for the parasites (Murase *et al.*, 1991). In the present study, we attempted to find out the optimum conditions for *in vitro* cultivation of *B. gibsoni* by examining the effect of several culture variables on the growth of the parasites. The present paper also reports the long-term *in vitro* cultivation of the parasites and the *in vivo* infectivity of the cultured parasites to dogs.

Materials and Methods

Babesia gibsoni strain

The strain was isolated from dogs infected with the parasites in Osaka Prefecture in 1985 and maintained by consecutive transfer into splenectomized dogs. This procedure for amplification of the parasites was repeated at about 3-month interval.

Culture medium

RPMI 1640 medium (Boehringer, Mannheim, Germany) was used. The medium was dissolved in 25mM 4-(2-hydroxy)-1-piperazine-ethansulfonic acid (Hepes) and supplemented with canine serum obtained from uninfected animals. The other supplements and their final concentrations were; 12mM $NaHCO_4$, penicillin G at 100 units per ml, streptomycin at 100 μg/ml and Fungizone (Whittaker Bioproducts, USA) at 250 μg/ml.

Preparations of canine red blood cells and serum

To prepare parasitized red blood cells, dogs were experimentally infected with *B. gibsoni* as described previously (Onishi *et al.*, 1990). The blood was collected into a flask containing glass beads, defibrinated and washed with phosphate-buffered saline (PBS, 10mM $NaHPO_4$-Na_2HPO_4 containing 155 mM NaCl, pH 7.3) as described previously (Onishi *et al.*, 1990). The parasitized cells were prepared from the animals 5 to 20 days after the experimental infection, because the red blood cells from chronic stage of the infection resulted in lower increase in the parasitemia rate in *in vitro* cultivation. The fresh red blood cells to exchange with

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those in medium for continuous cultivation system were prepared from uninfected dogs by the same procedure from the infected ones. Canine sera prepared by centrifuging the blood from 10 uninfected dogs were mixed and stored at –20°C until used.

Determinations of parasitemia and anemia

Parasitemia was assayed by counting the number of parasites in 1,000 red blood cells in Giemsa-stained thin blood smears and expressed as a percentage. Anemia was assayed by measuring packed cell volume (PCV) in automated particle counter (Model PC-608, Elma Co., Tokyo, Japan).

Cultural conditions

The incubation system for *in vitro* cultivation basically consisted of the parasitized red blood cells from experimentally *B. gibsoni*-infected dogs and RPMI-1640 medium (pH 7.3) supplemented with normal canine serum; the increase in parasitemia rate in RPMI-1640 medium was better than those in other media examined, e.g. α-MEM, M-119, F-12 and L-15. The parasites were cultured either in wells of 96-well (6.4 mm in diameter of a well) or in those of 24-well (16 mm in diameter of a well) plastic plate. The parasitized red blood cells and the nonparasitized cells were mixed at a ratio of 1:30 to 1:10 in a packed volume to make the parasitemia rate between 0.2 and 2.0%; this range of the rate did not affect significantly on the increase in the number of the parasites in the cultivation.

Results

To determine the optimum conditions, the cultivation was started with a mixture of red blood cells from infected and uninfected dogs at a ratio (v/v) of 1:10 in RPMI-1640 medium and the parasites were cultured for 7 days at 37°C by exchanging only the culture supernatant with fresh medium at every 24 h.

Fig. 1 shows the time course of the increase in the parasitemia rate at different serum concentrations. The parasitemia increased higher at 10 and 20% of the serum concentration than at 30 to 50%; in the media containing 40 and 50% of canine serum and also in that without serum, the growth of the parasites was rather inhibited. The serum was used without inactivation, because the growth of the parasites was inhibited in the medium containing the inactivated serum (Data not shown).

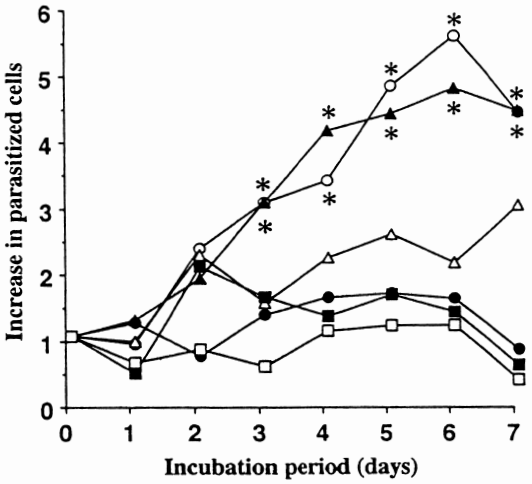

Fig. 1 Effect of different serum concentrations on *in vitro* growth of *B. gibsoni*. The serum was prepared from uninfected dogs as described in Materials and Methods and added into basal medium (pH 7.3), RPMI-1640, at the concentrations indicated. Parasitemia rate of the red blood cells used was 0.73%. Growth of the parasites was shown by a ratio of the number of parasitized cells at the incubation period indicated to that of the cells at the initiation of the cultivation. Mark (*) shows statistically difference from the others in nonparametric method at the 0.05% level. Symbols; ○, 10%; ▲, 20%; △, 30%; ■, 40%; □, 50% and ●, no serum added.

The increase in parasitemia rate was higher in gaseous environments of 5% CO_2 in air and 5% CO_2–10% O_2–85% N_2 than in those of 5% CO_2–2% O_2–93% N_2 and air only (Fig. 2). Total volume of the medium in a well of 96-well plate was 200 μl to keep a depth of 6.2 mm for a suitable oxygen supply.

By exchanging 100 μl of the culture supernatant with the same volume of fresh medium at every 24 h, the number of parasites increased higher than by exchanging at every 48 h and 72 h; the number of the parasites did not increase, when the medium was exchanged on 4th and 5th days after incubation (Fig. 3).

From the results described above, *B. gibsoni* was cultured *in vitro* at 37°C under a gaseous phase of 5% CO_2 in 200 μl of RPMI-1640 medium at pH 7.3 containing 20% canine serum in 96-well plate. The red blood cells of parasitemia rate between 0.2 and

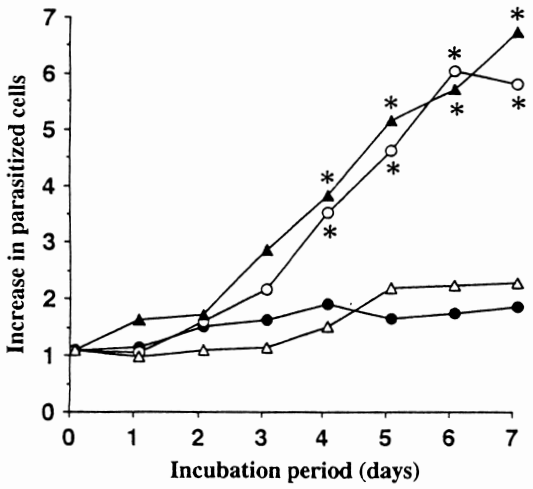

Fig. 2 Effect of gaseous environments on *in vitro* growth of *B. gibsoni*. The parasites were cultured at 37°C in RPMI-1640 medium containing 20% canine serum under gaseous environments indicated. Growth of the parasites was shown as described in legend to Fig. 1. Parasitemia rate of the red blood cells used was 0.75%. Mark (*) shows statistically difference from the others in nonparametric method at the 0.05% level. Symbols; ●, 5% CO_2–2% O_2–93% N_2; ○, 5% CO_2–10% O_2–85% N_2; ▲, 5% CO_2–95% air and △, in air.

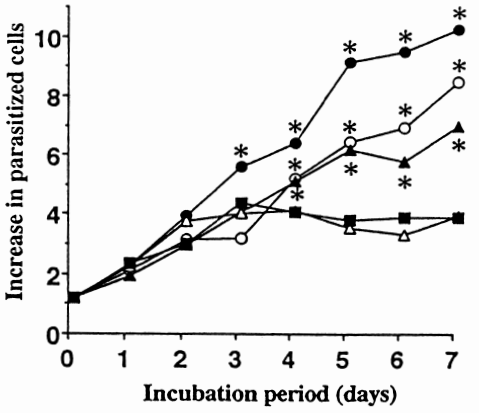

Fig. 3 Effect of intervals of medium exchange on *in vitro* growth of *B. gibsoni*. The parasites were cultured at 37°C in 200 μl of 20% canine serum-RPMI-1640 medium under 5% CO_2–95% air in flat-bottomed wells of 96-well plate by exchanging 100 μl of medium with fresh one at the intervals indicated. Growth of the parasites was shown as described in legend to Fig. 1. Parasitemia rate of the red blood cells used was 1.0%. Mark (*) shows statistically difference from the others in nonparametric method at the 0.05% level. Symbols; ●,24 h; ○, 48 h; ▲, 72 h; △, 96 h and ■, 120 h.

2.0% prepared from dogs 5 to 20 days after *B. gibsoni*-infection was mixed with canine red blood cells at a ratio of 1:10 and used as a source of parasites. The supernatant of the culture was exchanged at every 24 h with fresh medium.

Long term cultivation and the infectivity of B. gibsoni

To culture the parasites *in vitro* for longer period, the parasitized red blood cells was incubated in the medium under the conditions described above, except for that the parasites were cultured in 1.25 ml of the medium per well of 24-well culture plate (the depth of the medium was 6.2 mm), about 0.85 ml of the culture supernatant was exchanged every 24 h and also two third volume of the red blood cells was exchanged with the same volume of fresh canine red blood cells on every week after the incubation. During the first week, the parasites increased markedly, whereas those during the 2nd and 3rd weeks increased slightly and in 4th week the parasitemia rate decreased below 0.1% (Fig. 4). Within the first two weeks after the incubation, the major morphology of the parasites was ring forms, whereas those in dot forms increased thereafter and, at the end of the incubation, about 90% of the parasites changed into dot forms within the red blood cells.

To see whether the long term cultured *B. gibsoni* have a potency to infect to dogs, the parasites cultured for 28 days were injected intravenously into two splenectomized dogs at a dose of 4 ml red blood cell suspension containing about 1.5×10^5 parasites/ml. Fig. 5 shows the time course of parasitemia and anemia in one of two dogs after the infection. Both the increase in percentage of parasitemia and the decrease in hematocrit value were observed 2 weeks after the injection. The initiations of these changes delayed to those reported previously in experimental infection, which was induced by injecting $4–5\times10^7$ parasites/ml into dogs at a dose of 1 ml/kg (Onishi *et al.*, 1990). The results indicate that the long-term cultured parasites in dot forms still have a potency to cause a severe clinical disease in dogs.

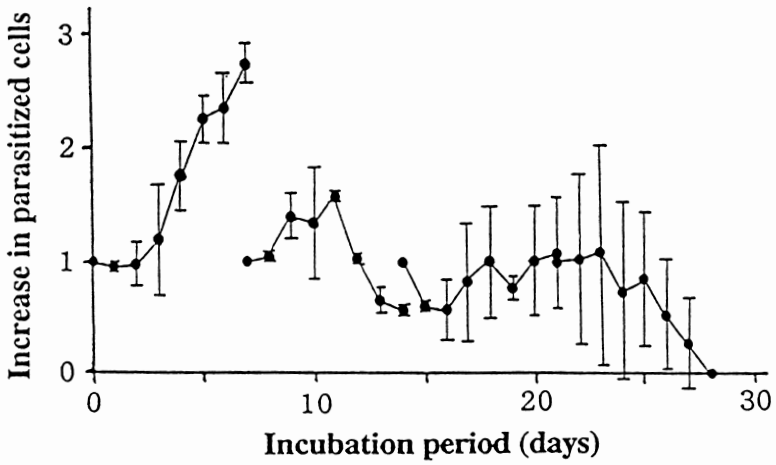

Fig. 4 Long-term cultivation of *B. gibsoni*. The parasites were cultured in a flat-bottomed well of 24-well plate. Of 1,250 μl medium in the well, 850 μl was exchanged at every 24 h after incubation and also one third of the red blood cells was exchanged at every one week after the cultivation; the red blood cells for the exchange were prepared from uninfected dogs as described in Materials and Methods. Growth of the parasites was shown as described in legend to Fig. 1. Parasitemia rate of the red blood cells used at the initiation of cultivation was 0.84%. Data indicate the mean ±SD.

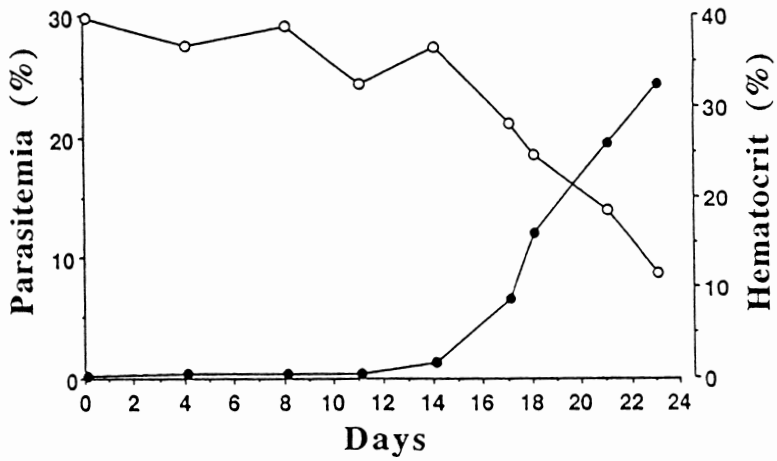

Fig. 5 Experimental infection by long-term cultured *B. gibsoni* in dogs.
B. gibsoni cultured *in vitro* for 28 days were injected into a splenectomized dog as described in the text. Symbols; ●, parasitemia and ○, hematocrit value.

Discussion

Several Babesia species have been successfully cultured *in vitro*. The continuous *in vitro* cultivation of hemoprotozoa was firstly reported with *Plasmodium falciparum* by Trager and Jensen in 1976. Since then, the method has been modified and applied to culture the other protozoan species *in*

vitro (Jack and Ward, 1980; Molinar *et al.*, 1982; Vega *et al.*, 1985; Vaayrynen and Tuomi, 1982). Most of these cultivation methods based on microaerophilous stationally phase system for *B. bovis* reported by Levy and Ristic (1980). The *in vitro* cultivation method for *B. gibsoni* reported by Murase *et al.* (1991) was also a modification of their system, in which the parasites were cultured in microtiter plate to supply oxygen efficiently during the cultivation. They cultured the parasites in α-medium for 14 days with a maximum percentage of parasitized red blood cells at 4.0% 7 days after incubation. In the present study, we firstly attempted to choose optimum conditions for short-term *in vitro* cultivation of *B. gibsoni*. Under the cultural conditions examined, RPMI-1640 medium was better than the other media for growth of the parasites and also the exchange of the medium on every 24 h was efficient to maintain high parasitemia rate. By the optimized cultural conditions in the present study, the maximum percentage of parasitemia obtained was as high as 9.5% after 7-day incubation (Fig. 3).

These cultural conditions for short-term cultivation was applied to long-term cultivation of the parasites, in which, in addition to the exchange of the medium, the red blood cells were also exchanged with fresh ones on every week, because most of red blood cells lysed during the incubation. By this subculture method, we could continue the *in vitro* cultivation of parasites for at least 28 days. However, the present system still may not be optimal for *in vitro* cultivation, because, in spite of supplying fresh medium and red blood cells, the number of parasites decreased gradually 1 week after the incubation and did not seem to grow after 4-week incubation. One possible explanations may be a limited availability of essential growth factor(s), which is supplied to the parasites in blood circulation. This was possibly implied by the fact that the growth of *B. gibsoni* was rather inhibited in the medium containing heated serum.

Morphological changes in *B. gibsoni* during the *in vitro* subculture were very similar to those in peripheral red blood cells of experimentally *B. gibsoni*-infected dogs as reported by Ninagawa *et al.* (1988). They showed that the major morphology of the parasites in early stage of the infection was ring form, whereas that of the parasites in later stage was dot form. By *in vitro* cultivation, though the period was for 2 weeks, Murase *et al.* (1991) also demonstrated similar changes in morphology of the parasites. These results suggest that the *in vitro* cultivation of *B. gibsoni* provides a simple means to solve the variable factors in morphological changes in *in vivo*, although the difference in life cycle between *in vitro* and *in vivo* multiplications should be solved.

The long-term *in vitro* cultured *B. gibsoni* caused severe anemia in dogs. The reproduced disease was very similar to that induced experimentally by injecting a large number of parasites, except for that lag period for 2 weeks was observed. These results indicate that the present method for long-term cultivation does not alter the infectivity of the parasites.

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