Multiplication of *Babesia gibsoni* in *In vitro* Culture and Its Relation to Hemolysis of Infected Erythrocytes

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*Babesia gibsoni* is a well-known blood parasite in the dog and causes hemolytic anemia in infected dogs [1, 3, 5]. The life cycle of the parasite, and the pathogenesis of the anemia, however, have not yet been fully elucidated. To clarify these problems in *in vitro* culture of the parasite seems to be needed, but it has not been successfully achieved until now. In the present study, *B. gibsoni* was successfully cultured in a settled layer of canine erythrocytes. Using this method for cultivation, we investigated the relationship between morphological changes in the intraerythrocytic parasites and hemolysis of erythrocytes during the cultivation.

*B. gibsoni* were cultured based on the method of Levy and Ristic [8] with some modifications. Defibrinated peripheral blood was obtained from *B. gibsoni*-infected dogs with a parasitemia of 1.0 to 8.2%. Infected erythrocytes were resuspended to a final packed cell volume of 10% in a culture medium consisting of 60% α-medium (Flow Laboratory, U.S.A.) supplemented with sodium pyruvate (0.11 mg/ml), glutamine (0.3 mg/ml), sodium bicarbonate (2 mg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml) and 40% normal dog serum. One volume of the suspension was then mixed with 9 volume of a normal erythrocyte suspension prepared from non-infected dogs. Two hundred microliters of mixed suspensions were placed in each well of 96-well flat-bottom microculture plates and incubated at 37°C under a humidified atmosphere of 5% CO₂ and 95% air for 15 days. The other peripheral wells of these plates received sterile water [4]. Every 24 hours, 100 μl of the culture supernatant was removed without disturbing the sedimented erythrocytes and replaced with an equal volume of fresh medium. Every 24 hours one well was sampled to examine the changes in the hemoglobin concentration of the supernatant, erythrocyte number, the percentage of parasitemia and the number of parasitized erythrocytes in the culture. Unparasitized cultures were treated as described above except no infected blood was used.

Figure 1 shows the changes of the parasitemia, the concentration of hemoglobin in the culture supernatant, and the number of erythrocytes during the cultivation. The parasitemia linearly increased and reached 4.0% on average at cultivation days 8 to 9, while its initial percentage of it was averaged 0.4%. After that, however, the percentage of parasitemia gradually decreased and it was less than 1% after 15 days. The number of erythrocytes in the culture decreased and the concentration of hemoglobin in the supernatant increased as the parasitemia increased. The number of erythrocytes decreased about 15% at cultivation day 6 in parasitized cultures, while it was unchanged in the unparasitized cultures until cultivation day 6. During cultivation days 10 to 15, however, the concentration of hemoglobin increased and number of erythrocytes decreased even in the unparasitized cultures. *B. gibsoni* organisms in the culture were morphologically classified into six forms on the basis of microscopical observations: the oval, dot, comma, pear, amoeboid, and petaloid forms (Fig. 2). When parasitemia reached the highest level during the incubation, most of the intraerythrocytic parasites were oval in form, though the other forms were occasionally observed (Fig. 3). In rare instance, several clusters of extracellular parasites were observed (Fig. 2g), indicating a rupture of erythro-

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Fig. 1. Changes in the parasitemia, the number of erythrocytes (RBC), and the concentration of hemoglobin in the culture supernatant (Hb) of parasitized culture (○) and unparasitized culture (▲). Each point represents the mean±S.D. of three experiments.
cyte parasitized with petaloid-shaped parasites. As parasitemia began to decrease, dot-shaped parasites dominated over the oval form, accompanied by slight increases in the percentages of comma, pear, and amoeboid forms. At the end of the incubation, almost all the parasites observed were a dot form. These results suggested that the oval-shaped parasites were in a multiplicative stage and that dot-shaped parasites might be a degenerative and/or inactivating type of the parasite. Other forms, commas, pears, and petaloids, might be in the process of division or schizogony, while the amoeboid form seemed to be a degenerate parasite form [7].

In general, babesia parasites invade erythrocytes in infected animals, resulting in the destruction of the host erythrocytes [2]. However, it has often been observed that severe regenerative anemia develops in spite of a very low parasitemia in infected animals [1, 3, 6], indicating an increase of destruction of nonparasitized erythrocytes. Our previous report suggested that the anemia induced by infection with B. gibsoni in dogs was partly due to an increased clearance of physiologically aged erythrocytes from the circulation by macrophages activated by the parasites [9]. In addition to this possibility, it has also been thought that nonparasitized erythrocytes might be injured by the parasites through an unknown mechanism. Onishi et al. [10] found the presence of a hemolytic factor in the serum of B. gibsoni-infected dogs. In the present study, hemolysis became prominent as the parasites multiplied in the culture. Furthermore, the number of erythrocytes decreased about 15% at cultivation day 6, in spite of the fact the highest parasitemia was only about 4% in the present culture. These results strongly indicated that nonparasitized erythrocytes were also destroyed in the parasitized cultures during multiplication of the parasites, and suggested that nonparasitized erythrocytes might be damaged by the multiplication of the parasites. What damage the parasites do, not only to parasitized erythrocytes but also to nonparasitized cells, will be studied in the future.

REFERENCES