A Short Term *in Vitro* Cultivation of *Babesia rodhaini* and *Babesia microti*

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ABSTRACT. *In vitro* cultivation of *Babesia rodhaini* (BR) and *Babesia microti* (BM) was attempted. When RPMI1640 was supplemented with 30 or 40% of non-treated fetal bovine serum (FBS), the gas mixture of 3% CO_2-8% O_2 best supported the growth of both parasites. Under this optimized condition, the percent parasitized erythrocytes peaked to approximately 4- and 2-times initial values for BR and BM, respectively. The cultivated parasites retained the infectivity to the host mice. BM showed the characteristic feature of division during cultivation. However, the lots of FBS will have to be taken into consideration, since the FBS lots were shown to give large varieties to the results. Selection of the appropriate FBS lot may yield the better growth of these protozoa.—KEY WORDS: *Babesia microti*, *Babesia rodhaini*, cultivation.

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In vitro cultivation of intraerythrocytic protozoa has long been a research objective for the production of merozoites as vaccine targets and for elucidating the mechanism of invasion into erythrocytes, as well as for the studies on metabolic characteristics of protozoa. Since Trager and Jensen [10] reported continuous development of human malaria *in vitro*, the cultivation method has been successfully applied to some species of *Babesia*, that is, *B. bovis* [7], *B. bigemina* [11], *B. divergens* [8], and *B. gibsoni* [9], but not to rodent species such as *B. rodhaini* (BR) and *B. microti* (BM).

Since the optimal gas mixture and serum concentration required in the *in vitro* culture largely varied among the protozoan species [7–11], atmosphere and serum were considered one of the most critical factors. Therefore, in this study the optimal condition that would give development of BR and BM *in vitro* was examined on the atmosphere and concentration of either non-treated or heat-inactivated serum.

Parasites: Babesia rodhaini (Australian strain) and *B. microti* (Munich strain) had been maintained in our laboratory by serial passages to 6 wk-old female ICR mice obtained from Charles River (Atsugi, Japan).

Cultivation procedure : Babesia-infected erythrocytes were collected with heparin from mice showing percent parasitemia of approximately 50% for both parasites. The plasma and buffy coat were removed after centrifugation at 1,700 g for 5 min at 4°C and washed with RPMI 1640 (pH 7.3, containing 20 mM Hepes, 100 U/m*l* penicillin, 100 μg/m*l* streptomycin). The percent parasitized erythrocytes (PPE) was adjusted to approximately 1% with erythrocytes obtained in the same way from uninfected mice, and washed again with RPMI1640. One part of the parasitized erythrocyte pellet was resuspended with 24 parts of RPMI 1640 (pH 7.30) supplemented with fetal bovine serum (FBS, either non-treated or inactivated by heating at 56°C for 30 min) at concentration of 10, 20, 30, or 40%. The 1.25 m*l* of this suspension per well was placed on 24-well tissue culture plates and incubated at 37°C in a humidified chamber (Sanyo, Tokyo, Japan) under various gas mixtures (3% CO_2 – 8% O_2, 5% CO_2 – 5% O_2, 5% CO_2 – 95% air). Other conditions such as the type of media (RPMI1640, M199, EMEM), pH of the culture (6.75, 7.0, 7.25, 7.5), animal species of serum source (bovine, mouse, human), initial percent of cell volume (4%, 10%, 20%) and PPE (approximately 1%, 5%, 10%, 25%) had been examined and selected in the preliminary study. The 0.8 m*l* of supernatant of each well was replaced with the identical volume of fresh media every 24 hr. The change of PPE was monitored daily on triplicate wells for each culture condition by the Giemsa-stained smears of the cultured erythrocytes.

Non-treated FBS: When the concentration of non-treated FBS was fixed to 10% or 20%, any of the 3 gas mixtures gave no increase of PPE in both BR and BM (data not shown). With 30% non-treated FBS (Fig. 1), however, PPE of BR peaked to approximately 4 times initial values (3.70%) at 48 hr after cultivation under 3% CO_2 – 8% O_2. The parasites at this time retained their initial morphology (Fig. 2-A, B) and also the infectivity as assessed by the inoculation into healthy mice. The culture under gas mixture of 5% CO_2 – 5% O_2 yielded much smaller increase of PPE and 5% CO_2 – 95% air was disadvantageous to the growth of BR. The 40% FBS gave essentially the same results as those with 30% (data not shown).

The PPE of BM kept increasing for 72 hr and reached the maximum value of 1.80% under 3% CO_2 – 8% O_2. Under this condition, the division of the parasites was most frequently observed. The parasite bodies newly formed by division were small and spindle shaped, apparently different from those merozoites at the initiation of culture, and accumulated within and sometimes outside the erythrocytes (Fig. 2-C, D). They closely resembled "maltese cross" form, which is known to be characteristic to BM merozoites *in vivo*. In addition, these parasites were confirmed infectious to mice. This suggested that the optimized culture condition obtained here may provide a simple means to solve the variable factors in morphological changes of BM *in vivo*.

Heat-inactivated FBS: When FBS was heat-inactivated and its concentration was fixed to 30%, 3% CO_2 – 8% O_2 was found to give better results in both BR and BM, as compared with the other gas mixtures. Even under this condition, however, no significant increase of PPE was

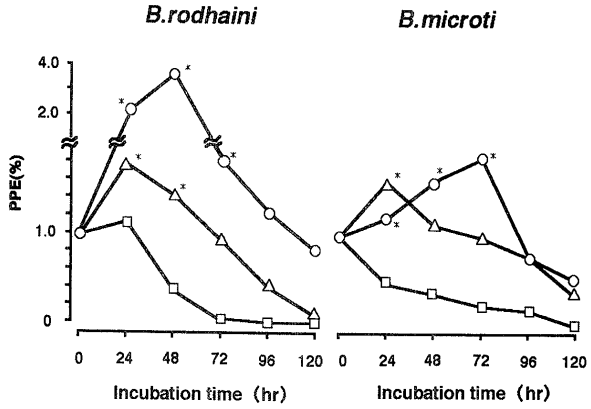

Fig. 1. Effects of different gas mixtures (—○—: 3% CO_2 — 8% O_2, —△—: 5% CO_2 — 5% O_2, and —□—: 5% CO_2 — 95% air) on the *in vitro* growth of *B. rodhaini* and *B. microti* in RPMI1640 containing 30% non-treated FBS. Each PPE represents the mean value of triplicate. *: Significantly ($p<0.05$) increased from initial PPE as determined by Student's *t* test.

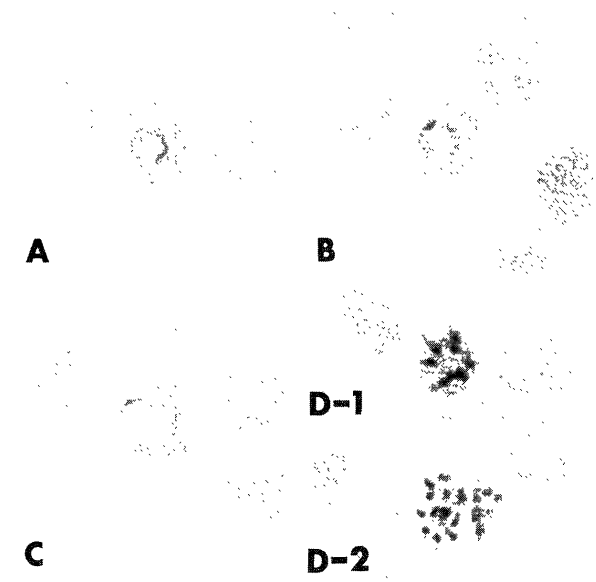

Fig. 2. *B. rodhaini* before (A) and 48 hr after (B) cultivation, and *B. microti* before (C) and 72 hr after (D) cultivation (Giemsa's-staining, × 1000). D-2 indicates the divided form of *B. microti* outside the erythrocytes.

observed (Fig. 3).

The lots of FBS: During further cultivation under the optimized condition of 30% non-treated FBS with 3% CO_2 – 8% O_2, the growth of these parasites were found to depend on FBS lots. Thus the effect of difference of FBS lots on the culture was examined. The increase of PPE in both BR and BM differed remarkably between 11 lots of FBS that was commercially obtained from several manufacturers (Fig. 4). The FBS lot that gave the largest increase of PPE was common to both protozoa. The similar results were

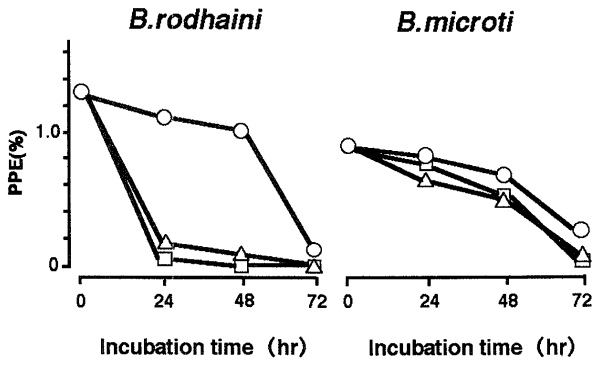

Fig. 3. Effects of different gas mixtures (—○—: 3% CO_2 — 8% O_2, —△—: 5% CO_2 — 5% O_2, and —□—: 5% CO_2 — 95% air) on the *in vitro* growth of *B. rodhaini* and *B. microti* in RPMI1640 containing 30% heat-inactivated FBS. Each PPE represents the mean value of triplicate.

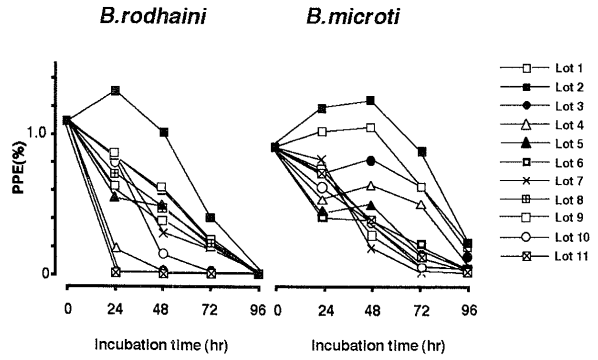

Fig. 4. Effects of different FBS lots on the *in vitro* growth of *B. rodhaini* and *B. microti*. The cultivation was carried out with 30% non-treated FBS under the gas mixture of 3% CO_2 — 8% O_2. Each PPE represents the mean value of triplicate.

obtained when additional 8 lots were examined (data not shown). Although the poor growth of BR and BM cultivated with heat-inactivated FBS (shown in Fig. 3) may imply the requirement of complement for the replication of these protozoa as has been reported in BR in human erythrocytes [1], a large variability of their growth with different lots of non-treated FBS suggests that some other factors are also critical in the *in vitro* growth of these parasites. Such factors may include purine nucleotides, especially hypoxanthine, of which uptake has been frequently utilized as the parameter of metabolic activity or infectivity of several protozoa *in vitro* [2, 4, 5, 6] and which is reported to improve the *in vitro* growth of human malaria [12]. Addition of hypoxanthine, however, to the culture of BR and BM showed no improvement (data not shown), in spite of its vigorous uptake by BR- and BM-infected mouse erythrocytes *in vitro* as reported previously [3, 4]. Therefore, some other bioactive components of FBS such as cytokines or hormones might be involved. The elucidation of those factors will contribute to establish the culture system that would support better development of

BR and BM.

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