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IN VITRO CULTURE OF *THEILERIA ORIENTALIS SERGENTI*, AND THE USE OF THE BOVINE-RED BLOOD CELL-SCID MOUSE MODEL IN-AID OF CULTIVATION

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ABSTRACT The *in vitro* culture of bovine *Theileria orientalis sergenti* and the suitability of the Bovine-RBC-SCID (Bo-RBC-SCID) mouse model in-aid of parasite cultivation are herein presented. Viable parasites were successfully maintained in culture at low oxygen atmosphere (5% O_2, 5% CO_2 and 90% N_2) for one month requiring three subcultures. Supplementation of RPMI 1640 medium with 40% adult bovine serum (ABS) gave the most satisfying 4-14.5% increase in parasitemia. From all indications, parasites in ABS-supplemented cultures, and those obtained from Bo-RBC-SCID mice appeared viable and normal. The parasite-specific p33 gene fragment was amplified in Bo-RBC-SCID mouse blood samples obtained at 30 days post-transfusion. To our knowledge, present data represent the first reported long-term cultivation of *T. o. sergenti in vitro* in tandem with the *in vivo* Bo-RBC SCID mouse system.

KEY WORDS: Adult bovine serum, Bo-RBC-SCID mice, *In vitro, Theileria orientalis sergenti*

INTRODUCTION

Spring outbreaks of theileriosis in grazing cattle in Japan have been attributed to *Theileria orientalis* [4]. Although theileriosis is associated only with onset of mild anemia, the disease may become severe and occasionally fatal in the presence of other infections and added stress due to animal shipment [21]. Much of the biological information about *T. orientalis* has been generated from experimental studies in cattle [5]. The first significant breakthrough in the cultivation of *Theileria* parasites was reported in 1945 [2]. Long term *in vitro* propagation of *Theileria annulata* schizonts [18], and continuous culture of *Theileria parva* in lymphoid cells [3, 19], and bovine cells [20] have been tried, and attempts to culture *T. orientalis* in bovine red blood cells (RBC) have generated insignificant merozoite invasion into host cells [6, 11]. *Babesia* sp. and *Theileria sergenti* have been successfully isolated from grazing calves in Japan via circulation of bovine RBC in SCID mice [10, 22-23]. Considering the economic importance of *Theileria* species and theileriosis in the livestock industry, and thus, the need for continuous and ample supply of parasites for experimental use, continuous search for a satisfactory culture system(s) is essential. In the present study, we

experimented on different culture conditions for *T. o. sergenti,* and evaluated the suitability of the Bovine-RBC-SCID mouse system in support of parasite cultivation.

MATERIALS AND METHODS

Parasite.

Bovine red blood cells (Bo-RBC) and SCID mice: *Theileria o. sergenti* (*Ikeda strain*) infected bovine blood [4] was provided by the National Institute of Animal Health (NIAH), Tsukuba, Japan. Bovine RBC were obtained from an uninfected adult Holstein cow at the university's animal farm, Obihiro, Japan. Blood was defibrinated and centrifuged at 800 *g* for 7 min, at 4°C, and the serum obtained was stored in aliquots at –30°C until use. RBC were washed with Vega y Martinez solution [24], three times at 800 *g* for 5 min, 4°C, and then stored at 4°C in the same solution. The buffy coat was completely removed to ensure non-contamination with leucocytes. Five to 10-wks old splenectomized C.B-17/ICR-scid/scid (SCID) (Japan Clea, Tokyo) mice were used in the transfusion of *T. o. sergenti*-infected Bo-RBC. Mice were kept in a conventional animal house and were fed and provided sterilized water ad libitum.

***Transfusion of SCID mice with T. o. sergenti*-infected Bo-RBC**: Transfusion protocol followed essentially that of Tsuji et al. [22]. Two ml of packed non-infected adult Bo-RBC were inoculated intraperitoneally (IP) into splenectomized SCID mice (Bo-RBC-SCID mice). A day after, these same mice were IP-inoculated with 2 ml of infected Bo-RBC (1% parasitemia), and were transfused 2 ml each of non-infected Bo-RBC, at 4 days interval. Parasitemia was monitored in Giemsa-stained blood smears. Blood from the Bo-RBC-SCID mice that had approximately 50% parasitemia was used in *in vitro* culture experiments.

Culture medium and sera: RPMI 1640 was supplemented with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), antibiotics (100 IU/ml penicillin, and 100µg/ml streptomycin), and pH adjusted to 7.0-7.2 with $NaHCO_3$, [1, 8, 9]. Sera used were fetal calf serum (FCS) (ICN Biomedical Japan Company), adult bovine serum (ABS) from an uninfected Holstein cow, and anti-*T. o. sergenti* serum (anti-TOS), generously provided by the NIAH, Tsukuba, Japan. FCS was obtained from a calf subcutaneously injected with *T. o. sergenti* tick-derived sporozoite suspension [17], and was heat-inactivated at 56°C for 30 min prior to use.

Culture preparation and monitoring: One ml of culture medium supplemented with either 40% or 100% of FCS, ABS or Anti-TOS was pipetted into 24-well plates. Per well, 40 µl of packed normal Bo-RBC, and 40 µl of *T. o. sergenti*-infected Bo-RBC obtained from SCID mice that registered approximately 50% parasitemia were seeded. Initial cultures were started at approximately 25% parasitemia, and subsequently administered daily replacement of one ml of fresh medium. Cultures were incubated at 37°C under low oxygen atmosphere (5% CO_2, 5% O_2, 90% N_2) or in 5% CO_2 in air. Parasitemia was noted using Giemsa-stained culture smears.

Inoculation of in vitro-cultured T. o. sergenti into SCID mice: To check the viability of the *in vitro*-cultured parasites and the capability of SCID mice to support parasite circulation and proliferation, another set of SCID mice were IP-injected with 2 ml of infected (1% parasitemia) Bo-RBC from cultures that had been maintained and subcultured *in vitro* for 10, 20 and 30 days. At 4 days interval, these same

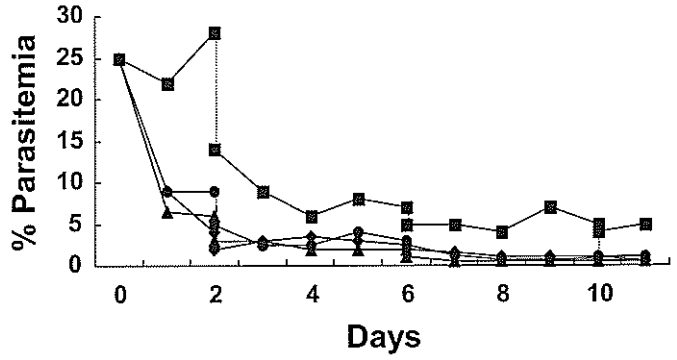

Fig. 1. Growth pattern of *T. o. sergenti* in RPMI 1640 with serum supplementation. (■) 40% adult bovine serum (ABS); (◆) 100% ABS; (●) 40% fetal calf serum (FCS); (▲) 100% FCS. Dotted lines indicate the start of subculture (i.e. replacement with fresh culture medium). Points represent mean daily percent parasitemia per 24-well plates. Note increase in parasitemia in 40% RPMI 1640 + 40% ABS supplementation.

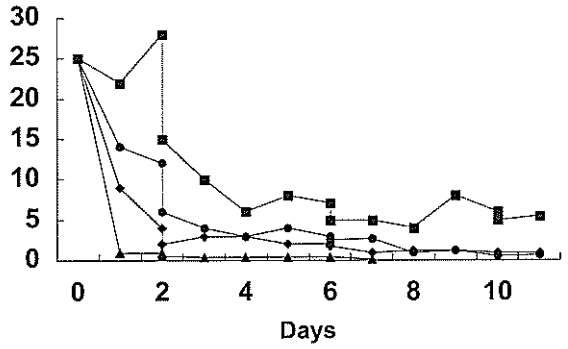

Fig. 2. Growth pattern of *T. o. sergenti* in RPMI 1640 with serum supplementation. (■) 40% adult bovine serum (ABS); (◆) 100% ABS; (●) 40% anti-*T. o. sergenti* serum (TOS); (▲) 100% anti-TOS. Dotted lines indicate the start of subculture (i.e. replacement with fresh culture medium). Points represent mean daily percent parasitemia per 24-well plates. Note increase in parasitemia in 40% RPMI 1640 + 40% ABS supplementation.

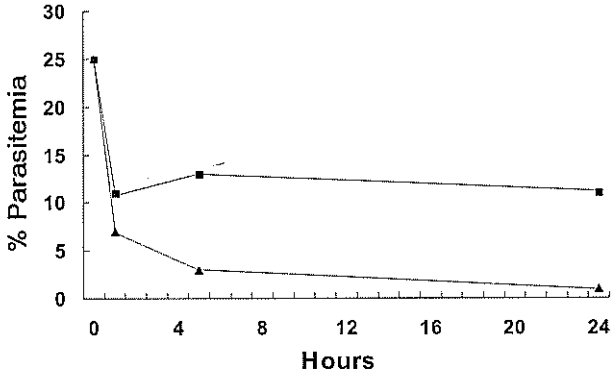

Fig. 3. Progression of parasitemia of *T. o. sergenti* during the first day of culture. (■) 100% adult bovine serum; (▲) 100% anti-*T. o. sergenti* serum (TOS). Points represent mean percent parasitemia per 24-well plates. Note significant ($p<0.05$) reduction in parasitemia in 100% anti-TOS- supplemented cultures.

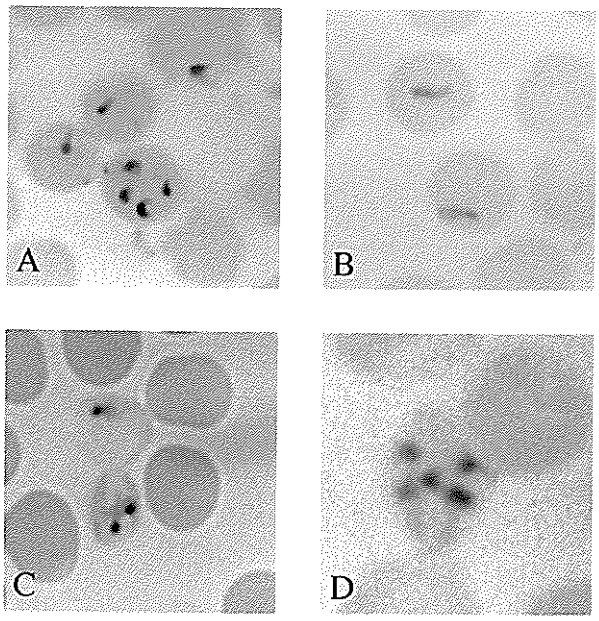

Fig. 4. Light micrographs of Giemsa-stained *T. o. sergenti*–infected Bo-RBC *in vitro*. A: RPMI 1640 + 40% adult bovine serum (ABS); B. RPMI 1640 +100% ABS; C: RPMI 1640 + 40% anti-*T. o. sergenti* serum (TOS); D. RPMI 1640 + 100% anti-TOS. Note pycnotic appearance of parasites (C, D).

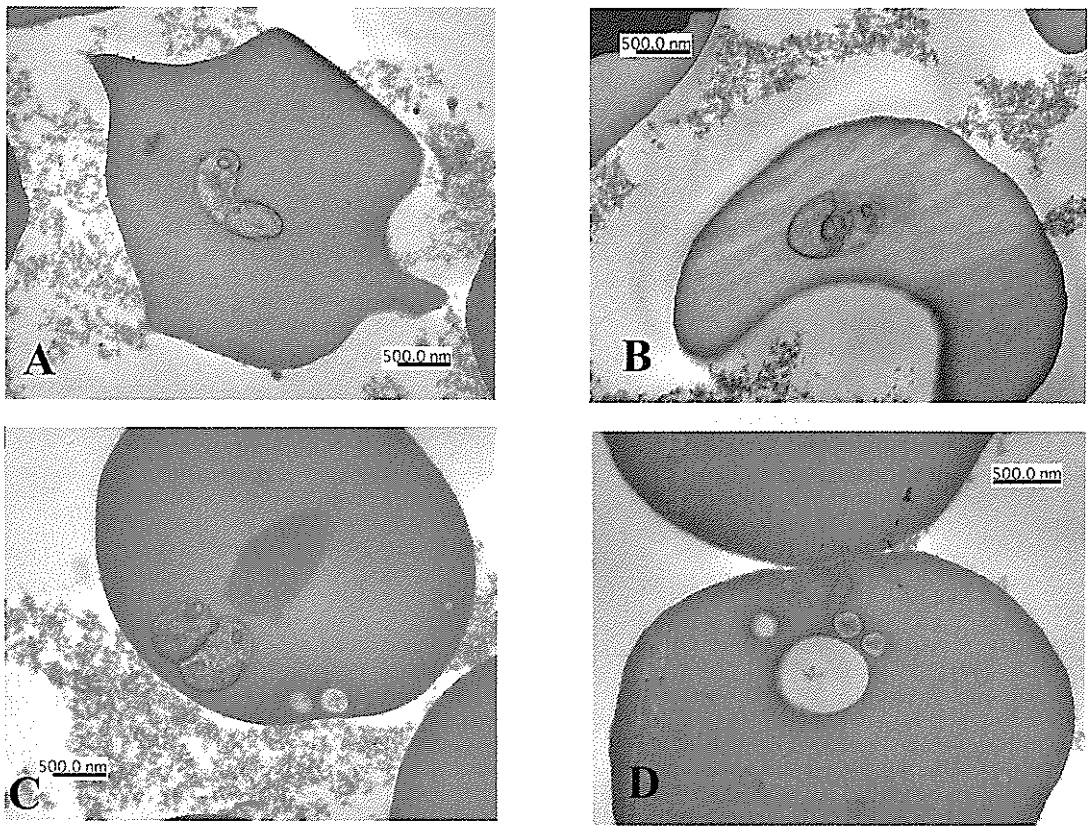

Fig. 5. Electron micrographs of *T. o. sergenti*-infected Bo-RBC. A. Bo-RBC from SCID mice; B: 5th hour of culture with 100% adult bovine serum.; C & D: 5th hour of culture with 100% anti-*T. o. sergenti* serum.

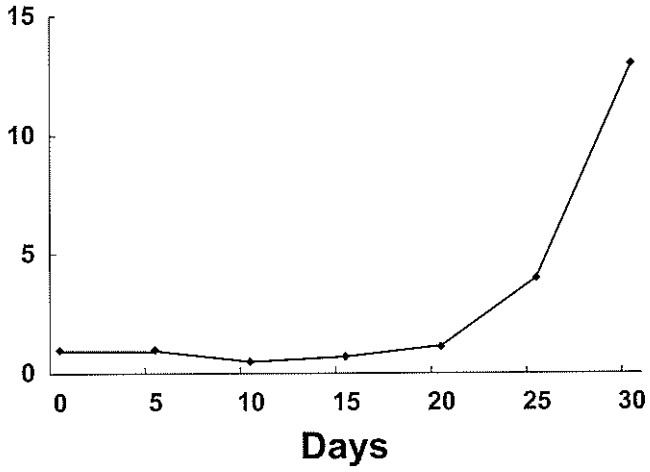

Fig. 6. Thirty days growth pattern of 10-days old transfused *in vitro*-cultured *T. o. sergenti* parasites to SCID mice. Points represent mean values from six splenectomized SCID mice. Each mouse was transfused normal B0-RBC at 4 days interval. Similar parasite growth pattern was noted in mice transfused with 20-days old parasite cultures.

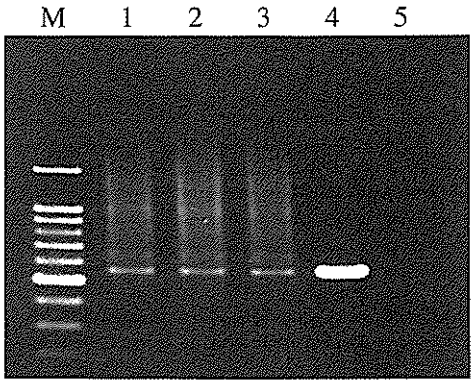

Fig. 7 Photograph of an agarose gel showing the product of the PCR amplification from cultured *T. o. sergenti* transfused into Bo-Scid mice. M;Marker, Lane 1;10th day, lane 2;20th day, Lane3;30th day, Lane 4;Positive control, Lane 5;negative control.

mice were then transfused 2 ml each of normal Bo-RBC. Parasitemia and parasite condition were noted using blood samples extracted from the mouse tail.

***Polymerase chain reaction (PCR)*:** To determine the circulation of transfused-infected Bo-RBC obtained from 10-, 20- and 30-days old parasite cultures in SCID mice, PCR-based marker assay was done using mouse tail blood samples at 30 days post-transfusion (PT). DNA samples were processed as previously described [16]. PCR amplification was performed in a 50 µl reaction mixture containing 40.5 µl of H_2O, 5 µl of 10x PCR buffer+Mg, 1 µl of 10 mM dNTP, 1 µl each of the oligonucleotide primers, 1 µl of Bo-RBC-SCID mouse blood sample, and 0.5 µl of Taq DNA polymerase [12]. The primers 5'-CGCATCAAGACACTCA-3', and 5'-GCCAAGCACTGTTCAT-3' were used to amplify the major p33/34 gene fragment [14-15]. The reaction mixture was subjected to 25 cycles with the following amplification conditions: denaturation at 94°C for 1 min, re-annealing at 58°C for 2 min, and amplification at 72°C for 3 min. Ten µl of the amplified products were checked using 1.5% agarose gel electrophoresis.

***Microscopic observation of culture condition and parasite morphology*:** Aliquots of 50 µl of the cultures were processed for light and transmission electron microscopy (TEM). Infected Bo-RBC-SCID mouse blood samples represented the control, while parasite condition, including proliferation was monitored in Giemsa-stained smears prepared from cultures. For TEM, cell pellets were left to coalesce at room temperature for 30 min, apportioned into smaller fragments, fixed in ice-cold 2.5% glutaraldehyde overnight at 4°C, and then post-fixed in 0.5% osmium tetroxide and 8% potassium ferricyanide in 50 mM cacodylate buffer with 50 mM $CaCl_2$, for 15 min on ice. Samples were stained with 2% aqueous uranyl acetate and dehydrated in acetone series, and thin sections were examined with a Hitachi H-7500 TEM (Tokyo, Japan).

***Statistical analysis*:** Differences in mean percent parasitemia were analyzed using the Student *t*- test at significance value $p<0.05$.

RESULTS

There was insignificant difference in the progression of parasitemia between cultures incubated at humidified atmosphere of 5% CO_2 in air, and at low oxygen atmosphere (5% CO_2, 5% O_2, 90% N_2). Culture cultivation at low oxygen atmosphere however, showed consistent pattern of parasite propagation exceeding 5% during the entire duration of experimentation (data not shown). Also, among several culture media initially evaluated for *T. o sergenti* cultivation, namely: RPMI 1640, M-199, DMEM, GIT, RPMI + M-199, RPMI + DMEM, and DMEM + M-199, containing 40% ABS supplementation, RPMI 1640 exhibited the highest parasitemia (data not shown). In the light of these preliminary findings, the RPMI 1640 medium and culture incubation at low oxygen atmosphere were selected for use in subsequent experiments, the results of which are presented herein.

Parasite propagation in cultures supplemented with 40% ABS (4-14.5% parasitemia) was significantly higher compared to cultures supplemented with 100% ABS, 40-100% FCS (Fig. 1), and anti-TOS (Fig. 2). The inhibitory effect of 100% anti-TOS supplementation on parasite survival was apparent as early as the 2nd day of cultivation, and on the 7th day, there was 100% parasite mortality (Fig. 2). As early as the 5th hour of culture, parasite survival in cultures supplemented with 100% anti-TOS was markedly

reduced (Fig. 3).

Majority of the parasites in ABS-supplemented cultures appeared normal with well-defined cell membrane compared to those parasites in 100% anti-TOS-supplemented cultures that were mostly non-viable and degraded (Figs. 4, 5). Some infected RBC in cultures supplemented with ABS showed marked contorsion (Fig. 5B). Likewise, transfused Bo-RBC in SCID mice demonstrated irregular cell shape (Fig. 5A), which apparently have not affected parasite viability, considering the parasite's sustained proliferation *in vitro*.

Transfused infected Bo-RBC into SCID mice showed no apparent increase in parasitemia during the first 15 days, but parasite density increased steadily thereafter, reaching peak parasitemia of approximately 15%, at 30 days PT (Fig. 6). Thirty days-old parasites *in vitro* culture were able to circulate in Bo-RBC-SCID mice for 40 days PT, but without apparent parasite proliferation (data not shown). *Theileria o. sergenti*-specific p33 gene fragment was detected in tail blood samples extracted from Bo-RBC-SCID mice at 10-, 20- and 30-days PT (Fig. 7).

DISCUSSION

In vitro cultivation of *T. sergenti* for 30 days (4-15% parasitemia), at low oxygen atmosphere (5% O_2, 5% CO_2 and 90% N_2), using RPMI 1640 medium supplemented with 40% ABS was achieved. Supplementation with 20% FCS has been shown to satisfy the development of *T. annulata* schizonts, however for long term cultivation, the use of either normal bovine or goat serum has been highly recommended [18]. Also, *T. orientalis* can be satisfactorily cultivated in 40% FCS-supplemented RPMI medium [6]. In the present study, despite the use of higher concentrations of FCS, the cultures performed unsatisfactorily. We think that the commercially-purchased FCS may have been lacking some factors or component(s) essential for parasite growth and survival.

Culture supplementation with anti-TOS markedly affected parasite survival and completely halted parasite proliferation. Infected RBC viewed with TEM had minute perforations similar to those earlier reported in *T. orientalis* [7], thought to be a prelude to subsequent parasite destruction. Interestingly, these perforations have been associated with recognition of the antigenic epitope(s) (i.e. initially the point of parasite entry into host cell) on the RBC surface membrane by antibodies and complement factors present in anti-*T. o. sergenti* serum [13, 25]. It would be interesting then to examine the mechanism(s) underlying this phenomenon in future studies.

The amplification of *T. o. sergenti*-specific DNA (p33 gene) in parasites obtained from SCID mice at 10, 20 and 30 days PT that corresponded to the 868 bp gene fragment in *Theileria* spp. [12, 15], implies that there was circulation of the parasite in Bo-RBC-SCID mice. We did not investigate the mechanism of erythrocyte circulation or substitution in SCID mice. However, in a related study by Tsuji et al. [22], SCID mice exhibited physical signs of anemia after the supply of *Theileria orientalis*-free Bo-RBC was stopped, and they postulated a parasite circulation route via the lymphatic system and a possible down-regulation of the formation of mouse RBC in the presence of sustained inflow of normal Bo-RBC into SCID mice. Also, they noted sustained proliferation of *T. orientalis* parasites in more than 30 SCID mice that lasted from one to nearly two months with peak parasitemia of 23-53.2%, contrasted with the 30 days duration and peak

parasitemia of about 15% obtained by us. We surmise that the difference in our findings may have been influenced by the cycling or passage of the parasite through a combined *in-vitro* and *in vivo* culture systems in the present study, as against continuous cycling of the parasite using only the Bo-RBC-SCID mouse model [22].

In conclusion, we successfully established a 30-day *in vitro* cultivation of *T. o. sergenti,* and demonstrated the usefulness of the Bo-RBC SCID mouse model in-aid of cultivation. The Bo-RBC-SCID mouse model may satisfactorily simulate conditions existing in the natural host(s) that are highly beneficial in sustaining long-term *in vitro* parasite survival and proliferation necessary for experimental use.

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