CONTINUOUS GROWTH OF BLOODSTREAM FORMS OF *TRYPANOSOMA BRUCEI BRUCEI* IN AN AXENIC CULTURE SYSTEM CONTAINING A LOW CONCENTRATION OF SERUM

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Abstract. An effective axenic culture system for bloodstream forms of *Trypanosoma brucei brucei* GUTat 3.1 containing a low concentration of serum is described. Bloodstream forms routinely maintained in Iscove's modification of Dulbecco's medium supplemented with 100 μM hypoxanthine, 30μM thymidine, 40μM adenosine, 1mM sodium pyruvate, 50 μM L-glutamine, 100 μM 2-mercaptoethanol and 20% FBS for more than one year were grown in the same medium supplemented with 5% FBS without reducing their growth rate. Then culture adapted trypanosomes in the culture medium containing 5% FBS were transferred into the modified medium supplemented with 0.5% FBS. For the constant growth of bloodstream forms in the medium containing 0.5% FBS, the culture medium was further supplemented with 200 μM L-alanine, 100 μM glycine, 10 μM L-oruithine hydrochloride and 10 μM L-citrullin. The trypanosomes propagated in this culture system for one year retained their infectivity for mice. This culture system was also shown to be useful for cloning of *T.b. brucei* GUTat 3.1 which is important for separation of mutants.

INTRODUCTION

The cultivation system for African trypanosome bloodstream forms using feeder layer cells was first reported by Hirumi *et al,* (1977). After development of their successful system many investigators tried to cultivate bloodstream forms of African trypanosomes using various kind of feeder layer cells (Hill *et al,* 1978a, b; Tanner, 1980; Burn *et al,* 1981, 1984; Balber, 1983; Yabu *et al,* 1983,1986; Yabu and Takayanagi, 1986, 1987). In 1985, two axenic culture systems for bloodstream forms were developed from independent standpoint by Duszenko *et al* (1985) and Balzt *et al* (1985). Baltzt *et al* (1985) showed that 2-mercaptoethanol (2-ME) is an essential growth factor for bloodstream forms of *T. b. brucei, T. equiperdum, T. evansi, T. b. rhodesiense,* and *T. b. gambiense.* Duszenko *et al* (1985) also indicated that cysteine secreted from feeder cells can act as a growth promoting factor and axenically cultured bloodstream forms of *T. b. brucei.* The major problem of their system was instability of added cysteine against oxidation during incubation at 37°C. Yabu *et al* improved their system by adding copper-specific chelating agent, bathocuproine sulfonate (BCS), and found it to be useful for cultivation of bloodstream forms of *T. b. gambiense* (1989) and *T. b. rhodesiense* (1990). Hirumi and Hirumi (1989) reported hybrid culture system of Duszenko *et al* (1985) and Balzt *et al* (1985) for bloodstream forms of *T. b. brucei* GUTat 3. 1 *in vitro.* Yabu (1993) also cultivated independently bloodstream forms of same clone of *T. b. brucei.* In the culture system, the essential growth promoting factor for bloodstream forms of this clone was 2-ME and in the absence of 2-ME, 100 μM cysteine and 10 μM BCS could not substitute 2-ME. However the reason of this dramatic difference between them is still unclear. Recently Hirumi *et al* (1997) demonstrated complicated and expensive serum-free culture for bloodstream forms of *T. b. brucei* and *T. evansi.* They cultured bloodstream forms in the medium supplemented with three serum derivatives and 15 defined components. We consider that establishment of an effective, stable and inexpensive long-term culture and cloning system for bloodstream forms of *T. b. brucei* is a useful tool for investigating of African trypanosomiasis. For the purpose we reduced the concentration of fetal bovine serum in the culture medium because the most expensive component of the medium supplement is fetal bovine serum. From our practical standpoint, we developed an effective, stable and economical culture system for bloodstream forms

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of *T. b. brucei* GUTat 3.1 containing a low concentration of serum.

MATERIALS AND METHODS

Trypanosomes

The pleomorphic clone of *Trypanosoma brucei beucei* GUTat 3.1 maintained in mice (dd strain) was used to initiate *in vitro* cultivation. This clone was kindly provided from Dr PR Gardiner, International Livestock Research Institute (ILRI), Nairobi, Kenya.

Culture medium for bloodstream forms

For the initial axenic cultivation of bloodstream forms, Iscove's modification of Dulbecco's medium (GIBCO BRL) was supplemented with 100 μM hypoxanthine (Wako Pure Chemical Inc, Osaka, Japan), 30 μM thymidine (Wako), 40 μM adenosine (Wako), 1 mM sodium pyruvate (Wako), 50 μM L-glutamine (Wako), 100 μM 2- mercaptoethanol (Katayama Chemical Industries Co, Ltd, Osaka, Japan) and 20% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories Inc, Logan, Utah, USA) as previously described (Yabu, 1993).

Axenic cultivation of bloodstream forms

Bloodstream forms collected from an infected mouse were diluted with the culture medium, described above, to 1 x 10^4 trypanosomes/ml. One ml of this suspension was placed into each well of 24-well tissue culture plates (Iwaki Glass, Tokyo, Japan), and incubated at 37°C under an atmosphere of 5% CO_2 and 95% air. After incubation for 24 hours, 200 μl of fresh medium were added to each well without removing the medium, thereafter 200 μl of fresh medium were changed every day. Three to 4 weeks later, the culture-adapted long slender bloodstream forms were transferred into 25 cm^2 tissue culture flasks (Iwaki Glass) containing 5 ml of culture medium and maintained therein.

Axenic cultivation of bloodstream forms in the medium containing a low concentration of serum

Bloodstream forms maintained in the culture medium containing 20% FBS for more than one year were suspended in the same medium supplemented with 5 % FBS to 10^4 trypanosomes/ml. One ml of this trypanosome suspension was added to 24-well tissue culture plates (Iwaki Glass) and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. After incubation for 24 hours, 200 μl of fresh medium were added to each well without removing the medium, thereafter 200 μl of fresh medium were changed every day. Cultured trypanosomes were counted by hemocytometer every 24 hours. Trypanosomes maintained in the culture medium containing 5% FBS for over one month (7 to 8 passages) were used for the cultivation in the medium containing 0.5% FBS. For the constant growth of bloodstream forms in the culture medium supplemented with 0.5% FBS, culture medium described above was further supplemented with 200 μM L-alanine, 100 μM glycine, 20 μM L-ornithine hydrochloride, and 10 μM L-citrullin. These chemicals used were purchased from Wako Pure Chemical Inc. Bloodstream forms were suspended in the modified medium supplemented with 2.5 to 0.1% FBS to 5 x 10^4 trypanosomes/ml and 1 ml of the trypanosome suspension was added into 24-well tissue culture plates (Iwaki Glass) and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After incubation for 24 hours, 200 μl of fresh medium was added to each well without removing the medium, thereafter 200 μl of culture medium was changed every day. After one month (7 to 8 passages) culture-adapted long slender bloodstream forms in the culture condition containing 0.5% FBS were transferred into 25 cm^2 tissue culture flasks (Iwaki Glass) containing 5 ml of culture medium and maintained therein. Every 10^{th} day, a group of five mice (dd strain) were inoculated ip with 1 x 10^4 trypanosomes that had been cultured in the medium with 0.5% FBS to determine their infectivity and virulence of the trypanosomes for mice.

In vitro cloning

One month after initiation of the culture containing 0.5% FBS using 25 cm^2 tissue culture flasks, *in vitro* cloning of bloodstream forms was made by means of limiting dilution method using 96 well tissue culture plates (Yabu *et al,* 1989). Cultured bloodstream forms were diluted with fresh medium to 1 x 10^3 trypanosomes/ml, and 0.5 μl of suspension was added into 96 well tissue culture plates (Iwaki Glass). The size of each drop of trypanosome suspension was smaller than a microscope field (10

x 10 magnificatiton). Each well containing a single trypanosome was given 100 µl of culture medium. The growth of trypanosomes was evaluated in each well every 24 hours by counting the bloodstream forms under phase-contrast microscope.

RESULTS AND DISCUSSION

Bloodstream forms of *Trypanosoma brucei brucei* GUTat 3.1 could be maintained constantly in Iscove's modification of Dulbecco's medium supplemented with 100 µM hypoxanthine, 30 µM thymidine, 40 µM adenosine, 1 mM sodium pyruvate, 50 µM L-glutamine, 200 µM L-alanine, 100 µM glycine, 20 µM L-ornithine hydrochloride, 10 µM L-citrullin, 100 µM 2-mercaptoethanol, and 0.5% FBS at 37°C. In this culture system minimum concentration of serum required for continuous growth of bloodstream forms was 0.5% and in the absence or lower concentration than 0.5%, trypanosomes died out within 24 hours. For the constant growth of trypanosomes in the culture medium containing 0.5% FBS, bloodstream forms required two step reduction procedures of serum concentration, from 20 to 5% and then 5 to 0.5%. As shown in Fig 1, bloodstream forms propagated in the culture medium supplemented with 20% FBS for more than one year could be grown in the same medium containing FBS ranging from 20 to 5% without reducing their growth rate. Under these culture conditions, bloodstream form populations increased in number up to 2 to 3 x 10^6/ml by day 4 to 5 after initiation of the culture. In the first step of serum concentration reducing procedure, the minimum serum concentration for continuous growth of bloodstream forms was 5%. The trypanosomes could be maintained in the culture conditon containing 5% FBS for more than one year. For continuous cultivation in the culture medium containing serum less than 5%, trypanosomes had to be maintained in the culture medium supplemented with 5% FBS for more than one month (7 to 8 passages). To obtain the constant growth of bloodstream forms in the culture medium containing serum lower than 0.5%, culture medium was further supplemented with 200 µM alanine, 100 µM glycine, 10µM ornithine hydrochoride and 10 µM cetrullin. In the culture medium containing 2.5 to 0.5% FBS, trypanosomes increased in number to 1.5 to 1.6 x 10^6/ml by day 4 after initiation of the culture (Fig 2). This maximum

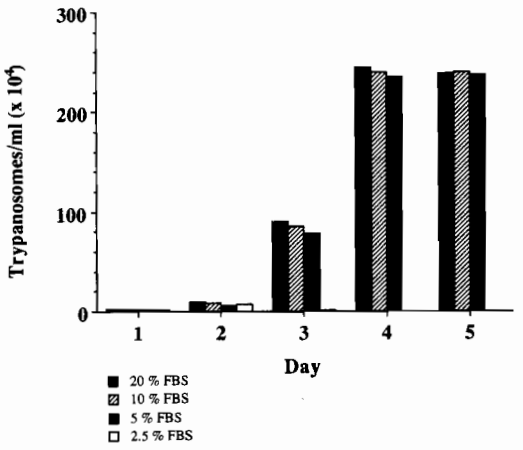

Fig 1–Growth of bloodstream forms of *T. b. brucei* GUT at 3.1 in the medium containing 20 to 2.5% FBS. The bloodstream forms (1 x 10^4/ml) maintained in the culture medium containing 20% FBS for more than one year were cultured in 24-well tissue culture plates in the medium containing from 20 to 2.5% FBS at 37°C. The results represent the means of five different experiments.

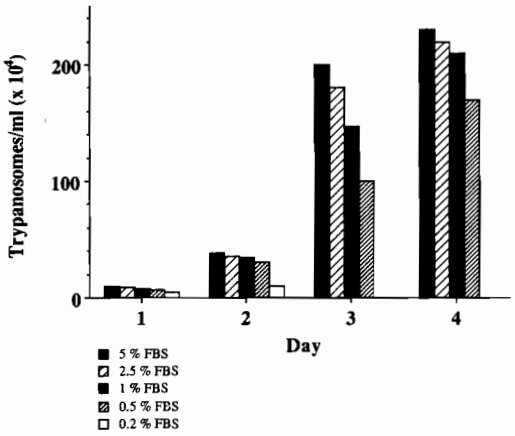

Fig 2–Growth of bloodstream forms of *T. b. brucei* in the modified medium containing 5 to 0.2% FBS. The bloodstream forms (5 x 10^4/ml) maintained in the cultured condition containing 5% FBS for one month were cultured in 24-well tissue culture plates in the modified medium supplemented with from 5 to 0.2% FBS at 37°C. The results represent the means of five different experiments.

cell density in the medium containing 0.5% FBS was almost same as that seen in an axenic culture supplemented with 20% FBS, previously reported (Yabu, 1983). When bloodstream forms were cultured in the medium containing 0.2% FBS, the trypanosome populations decreased and all populations died by day 3. These results indicate that the minimum concentration of serum required for continuous growth of bloodstream forms of this clone is 0.5%. For long-term culture, the trypanosome suspensions (5 x 10^4/ml) which have been cultured in 24-well tissue culture plates for one month (7 to 8 passage) were transferred into 25 cm^2 tissue culture flasks and maintained therein. The bloodstream forms were continuously maintained as long slender forms in this culture condition for more than one year and no change in infectivity for mice was observed after long-term cultivation (data not shown). All mice inoculated with bloodstream forms (1 x 10^4 trypanosomes) of *T. b. brucei* which had been cultured for one year became parasitemia on day 4 and, thereafter, successive waves of parasitemia were seen in infected mice. One month after initiation of the culture containing 0.5% FBS using 25 cm^2 tissue culture flasks, *in vitro* cloning of bloodstream forms was done by means of limiting dilution method using 96 well tissue culture plates (data not shown). The growth of trypanosomes was assessed in each well every 24 hours by counting under phase-contrast microscope. This culture system was also useful for cloning of bloodstream form of *T. b. brucei* GUTat 3.1 which is important for separation of mutants.

The axenic culture system containing a low concentration of serum demonstrated here is economical, extremely useful for long-term culture and cloning for bloodstream forms of *T. b. brucei* GUTat 3.1 which is available for many investigators. Furthemore, this axenic culture system might be an effective tool for investigating a wide-range of immunological and biological characteristics of this clone of *T. b. brucei* and possibly of other clones of African trypanosome bloodstream forms *in vitro*.

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