THE SUCCESSFUL SYSTEM IN LONG-TERM CULTIVATION OF *TRYPANOSOMA GAMBIENSE* BLOODSTREAM FORMS

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INTRODUCTION

Trypanosoma gambiense, an important pathogenic protozoan parasite which cause African sleeping sickness in man, occur only as bloodstream forms in the host. Recently, the conditions necessary for effective long-term cultivation of African trypanosome bloodstream forms have been studied for *T. brucei* (Hirumi *et al.*, 1977; Hill *et al.*, 1978a; Brun *et al.*, 1981), *T. rhodesiense* (Hill *et al.*, 1978b; Brun *et al.*, 1981; Balber, 1984) and *T. gambiense* (Brun *et al.*, 1981; Balber, 1983; Yabu *et al.*, 1983). The cell line used as feeder layers was one of the most important factors which influenced the growth properties of trypanosomes. Until now, a few cell lines were reported to be available for *in vitro* cultivation of parasites as feeder layers.

Exploitation of new cell lines for further progress of *in vitro* cultivation systems for *T. gambiense* bloodstream forms would be desirable to provide effective tools for establishing rapid drug-screening systems and investigating a wide range of immunological and biochemical characteristics of trypanosomes.

The present study describes and shows that rat astroglioma cell line (GA-1) has the ability of supporting growth and is suitable for the long-term culture of bloodstream forms of *Trypanosoma gambiense*.

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MATERIALS AND METHODS

Parasites: Wellcome strain, *T. gambiense* (antigenically O-type) maintained in 18-20 g mice (dd strain) serially transferred at 3 day intervals, and parasite populations frozen 6 months earlier in liquid nitrogen with 10% dimethyl sulfoxide (DMSO) were used for the initial cultivation.

Culture medium: Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco Co.), buffered with 25 mM 4-(2-hydroxyethyl 1)-1-piperazineethanesulfonic acid (HEPES, Dojin Chemical Co.), was used for the cultivation of *T. gambiense* bloodstream forms. The pH was adjusted to 7.2 with 4N NaOH. The osmolarity was adjusted to 300 milliosmole/kg. The medium was sterilized by Millipore filter (0.22 μm), and supplemented with 20% heat-inactivated Wistar rat, human, rabbit, new born calf (Gibco Co.) and fetal calf sera (Gibco Co.). Antibiotics used were penicillin (100 IU/ml), streptomycin (100 μg/ml), and kanamycin (100 μg/ml). All chemicals were purchased from Meiji Seika Co.

Feeder layer cells: Astroglioma cells (GA-1, malignantly transformed in the secondary culture of Wistar rat glioblasts in the presence of an adhesive comprising epoxy compound, kindly provided by Dr. T. Kato, Dept. of Biochemistry, Nagoya City University Medical School; Kato *et al.*, (1984) were used as feeder layer cells. They secreted growth-promoting factors for glioblast cells into the culture medium. Cells were subcultured at 4-day intervals in plastic tissue culture flasks (Falcon Co., No. 3013, 25 cm^2) in RPMI 1640

medium supplemented with 20% heat-inactivated fetal calf serum.

Culture of trypanosomes: Heparinized heart blood containing parasites was removed from mice in their log phase of growth and cryopreserved parasites were diluted with the culture medium to a density of 5×10^4 parasites/ml, and 5ml of these trypanosome suspensions were added to each 25 cm^2 tissue culture flask in which GA-1 cells were pre-seeded for 24 hours and incubated at 37°C in an atmosphere composed of 5%CO_2 and 95 % air. After incubation for 24 hours, 1 ml of fresh culture medium was added into the flasks, and thereafter 1 ml of culture medium was removed every day. Propagated trypanosome populations were counted by hemacytometer every 24 hours. The population doubling time (PDT) of *in vitro* cultured trypanosomes was determined according to the method described by Hirumi *et al.*, (1980). Every tenth day, a group of five mice (dd strain) were inoculated intraperitoneally (ip) with 1×10^4 trypanosomes which had been cultivated *in vitro* in order to determine their virulence for mice.

In vitro cloning: Thirty days after initiation of the culture, *in vitro* cloning was made by the limiting dilution method using 96 well tissue culture plates (Costar Co., No 3596, 0.32 cm^2/well). The culture medium was discarded using a sterile pipette and then 0.2 ml of parasite suspension (0.5 trypanosome/ml) was added to each well containing 1×10^4 GA-1 cells preseeded for 24 hours. After incubation at 37°C for 3 hours, each well was examined minutely for the presence of parasites under a phase-contrast microscope. After incubation for 24 hours, each well containing a single trypanosome was given 0.1 ml of fresh culture medium; thereafter 0.1 ml of culture medium was exchanged every 48 hours. When the trypanosome populations increased to more than 200 parasites per well on day 3, the parasites were transferred into 25 cm^2 tissue culture flasks containing GA-1 cells. Every 10 days after cloning, the antigenic types of each clone established were determined by means of the indirect immunofluorescent staining method (Doyle *et al.*, 1980).

Preparation of mouse anti-trypanosome serum against *in vitro* cloned parasites: In order to obtain the test antiserum for each cloned parasite, mice were infected ip with 1×10^4 cloned trypanosomes. Seventy-two hours after inoculation, the mice were given 1 ml of fresh normal human serum ip to cure the trypanosome infection completely (Inoki, 1952; Osaki, 1959; Takayanagi *et al.*, 1974). Fourteen days later, the mice were exsanguinated by heart puncture. Pooled sera with an agglutination titer of 1 : 16 were stored at -80°C and used for immunofluorescent test as anti-cloned type trypanosome serum.

Transmission electron microscopy: Coverslips seeded with GA-1 cells and exposed to *in vitro* cultured *T. gambiense* in culture flasks for 24 hours were fixed in 1 % glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB), pH 7.5, on ice for 1 hour. Fixed specimens were washed three times with cold 0.1 M phosphate buffered saline (PBS), pH 7.5. The specimens were then postfixed in 1 % (w/v) OsO_4 in PBS for 1 hour on ice and subsequently dehydrated in an ascending series of acetone, and embedded in Epon 812. Sections were double stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12 electron microscope.

Scanning electron microscopy: For scanning electron microscopic examination of *in vitro* cultured *T. gambiense* bloodstream forms in the presence of GA-1 cells, coverslips seeded with GA-1 cells and exposed to trypanosomes in culture flasks for 24 hours were fixed in 1 % glutaraldehyde in SCB. After 1 hour at 4°C, they were washed 3 times with PBS, and then postfixed in 1%

(W/V) OsO_4 in PBS for 1 hour. Dehydration was carried out in acetone. After dehydration, they were dried by a critical point dryer HCP-1 (Hitachi Co.). A DS-130 scanning electron microscope was used (Akashi Seisakusho Co.).

RESULTS

Trypanosoma gambiense trypomastigote bloodstream forms could be continuously grown at 37°C in 25 mM HEPES-buffered RPMI 1640 medium (pH 7.2, 300 milliosmole/kg) supplemented with 20% heat inactivated fetal calf serum in the presence of GA-1 cells as feeder layers (Fig. 1). Cultures were initiated with parasites from both infected blood of mice and cryopreserved populations. There was no significant difference between them in the propagation of parasites *in vitro*.

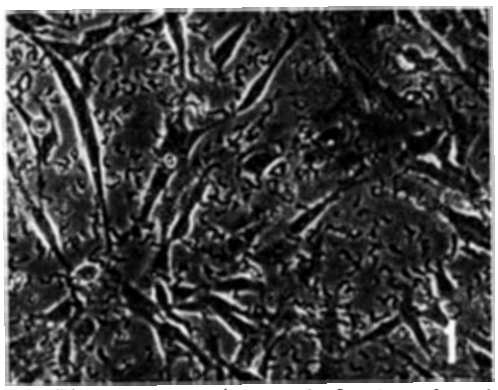

Fig. 1—Phase-contrast micrograph of *in vitro* cultured *T. gambiense* in the presence of GA-1 cells. (× 200).

When cultured *T. gambiense* were transferred into the culture medium containing 20% of conditioned medium from GA-1 cells, in which feeder layer cells had secreted growth-promoting factors, in the presence of GA-1 cells fixed with 1% glutaraldehyde at 4°C for 1 hour, the parasite populations did not demonstrably increase in number during the first 12 hours and died out within 24 hours. A similar result was obtained in the culture medium containing the extract of GA-1 cells (1×10^6 cells/ml).

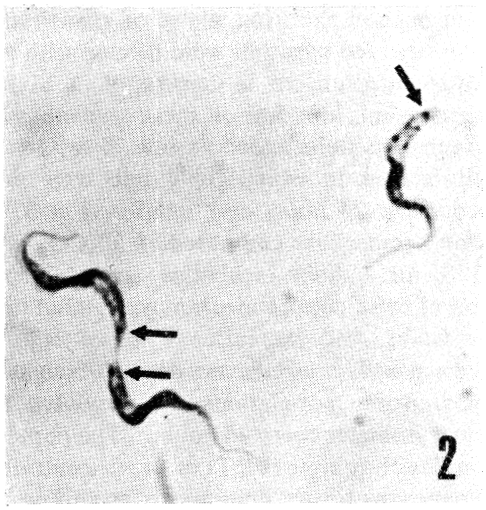

Fig. 2—Giemsa-stained smear prepared from *T. gambiense* cultured *in vitro* for 100 days. The parasites were long slender forms and kinetoplast (arrows) could be recognized at the extreme posterior ends. (× 1200).

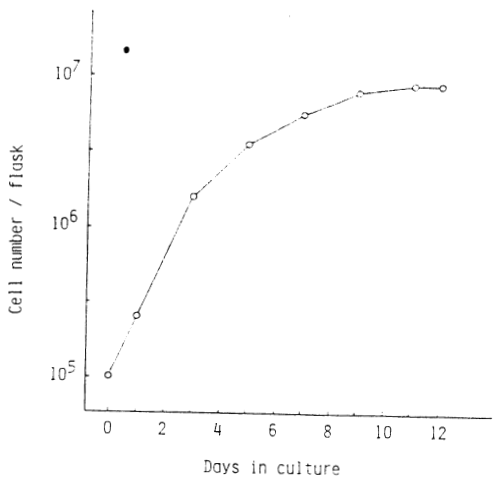

Fig. 3—Growth curve of GA-1 cells. Cells were seeded in 25 cm^2 tissue culture flasks under RPMI 1640 medium supplemented with 20% inactivated fetal calf serum.

There was a close relationship between the seeding density of feeder layer cells and the growth rate of trypanosomes (Fig. 3). The best growth rate was obtained in culture flasks in which trypanosome populations (5×10^4/ml) were inoculated into the culture medium containing 1×10^6 GA-1 cells pre-seeded for 24 hours. The trypomastigote form populations increased in number up to $7\text{-}8 \times 10^6$ trypanosomes/ml by day 3 after initiation of the culture by exchanging one-sixth of the culture medium daily. When trypanosomes were cultured without exchanging the medium parasite populations increased in number during the first 48 hours, reaching a maximum, density of 2×10^6 trypanosomes/ml on day 2 and thereafter decreased rapidly. All the populations died out by day 4 (Fig. 4). The population doubling time (PDT) of trypanosomes in this culture condition was 7-8 hours during the first 24 hours. This PDT PDT was similar to that seen in mice.

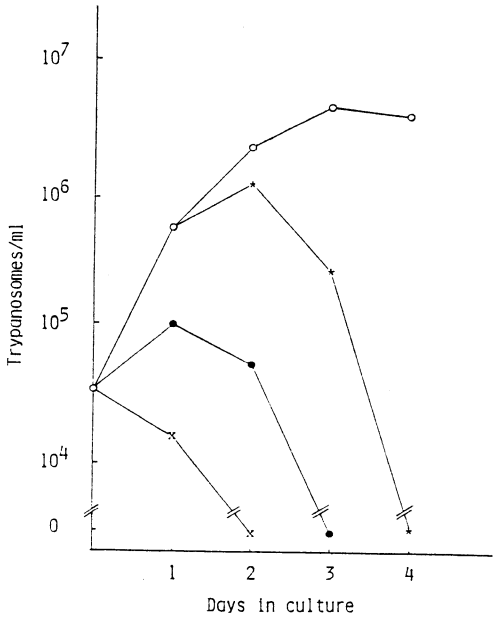

Fig. 4—Growth of *T. gambiense* bloodstream forms at 37°C in 25 cm^2 culture flasks in the presence of GA-1 cells initiated with 3 different seeding densities in RPMI 1640 supplemented with 20% inactivated fetal calf serum *in vitro*. One-sixth of the culture medium was exchanged every 24 hours.
GA-1 cells/flask: —o— 1×10^6, —●— 2.5×10^5, —x— 5×10^4, —*— $\times 10^6$, no medium change.

For the long-term cultivation, the trypanosome populations (5×10^4/ml) were transferred into the new culture flasks with GA-1 cells when their number increased up to $3\text{-}5\times10^6$/ml. In this culture system, the populations were continuously maintained for more than 200 days. Majority of the trypomastigote forms were in suspension, and 15-20% of trypanosomes attached to the surface of GA-1 cells, and actively multiplied. Attachment of trypanosomes to the surface of GA-1 cells was observed within 2 hours after inoculation into new culture flasks. The rate of attachment of parasites to the surface of GA-1 cells was constant throughout the cultivation. The adherence and multiplication of parsites were clearly revealed by both transmission and scanning electron microscopy (Figs. 5-6).

There was no change in the virulence of populations against mice after the long-term cultivation. All the mice inoculated with 1×10^4 trypanosomes which had been cultured for 200 days, showed parasitemia on day 3, and died within 5 days.

Heat-inactivated rat, rabbit, new born calf and human sera as the medium supplement were tested for their capacity to support the growth of parasites *in vitro* instead of fetal calf serum, none of them had growth promoting capacity. Moreover, human serum possessed a lytic property. All the trypanosomes died out within 3 hours with incubation at 37°C in the medium supplemented with 20% heat-inactivated human serum in the presence of GA-1 cells.

Thirty days after initiation of the culture, *in vitro* cloning by means of the limiting dilution method was performed with 96 well tissue culture plates containing GA-1 cells.

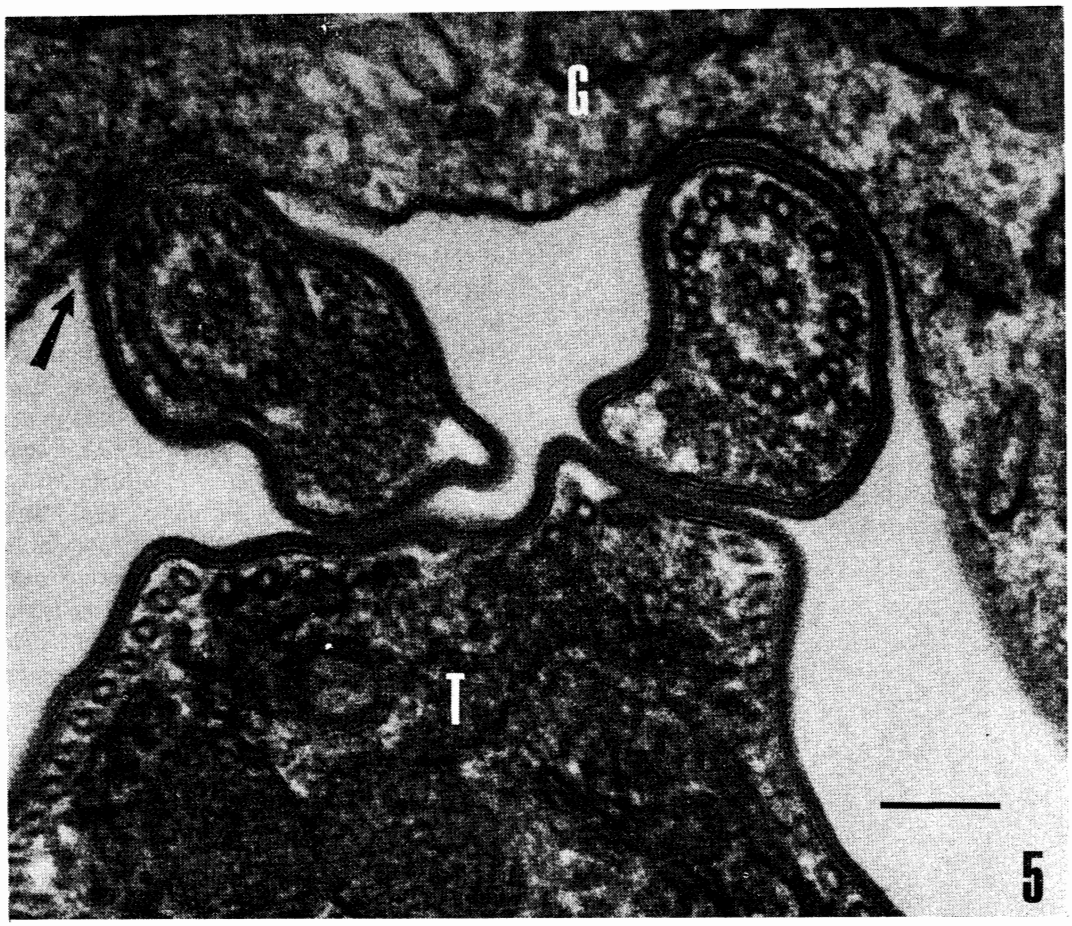

Fig. 5—Transmission electron micrograph of ultra thin section of *in vitro* cultured *T. gambiense* bloodstream forms in the presence of GA-1 cells at 37°C for 80 days. Surface coat is evident on the cell surface and flagella membrane. The arrow shows attachment of parasite to the surface of GA-1 cell. Section stained with uranyl acetate and lead citrate. G, GA-1 cell; T, trypanosome. (× 135,000) bar, 0.1 μm.

The multiplication of parasite was assayed in each well every 24 hours by phase-contrast microscope. As shown in Table 1, GA-1 cells were useful for cloning *T. gambiense in vitro*. Cloned populations established were tested for determination of their antigenic types and resistance to heat-inactivated human serum. As shown in Table 1, there was no variant population as far as tested. All the clones isolated were antigenically O-type, and no development of new variant types could be detected even in more 30-day cultivation subsequent to initial cloning by mean of the indirect immunofluorescent staining method. Resistant parasite to inactivated human serum did not appear in such clone populations. In Giemsa-stained preparations, most of the parasites in long term cultures were long slender forms, and kinetoplast could be recognized at the extreme posterior ends (Fig. 2). A surface coat could also be demonstrated by transmission electron microscopy (Fig. 5).

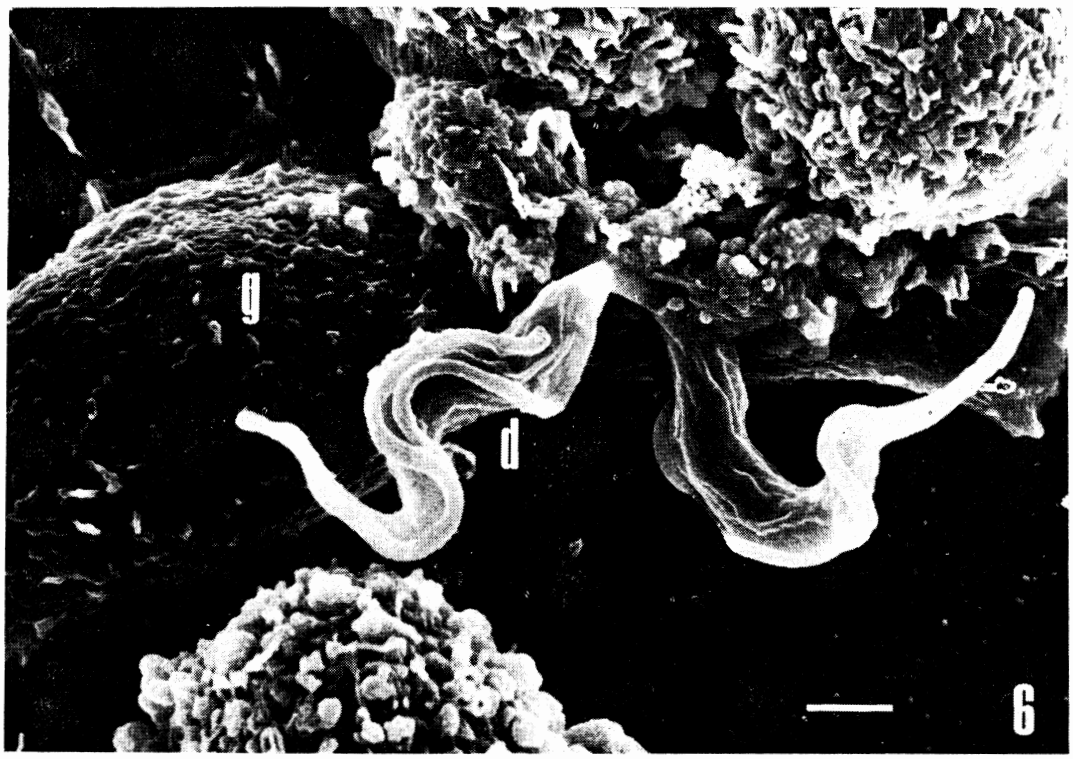

Fig. 6—Scanning electron micrograph of *in vitro* cultured *T. gambiense* bloodstream forms grown with GA-1 cells at 37°C for 60 days. g, GA-1 cell; d, a dividing form. (× 4,700) bar, 2 μm.

Table 1

Clones of *T. gambiense** propagated *in vitro*.

	Number of trypanosomes/well			
Days in culture	1	2	3	
Established clones				Antigenic types
B5	5	67	>500	0
C10	8	142	>500	0
E6	5	38	200	0
F3	7	75	>500	0
G7	5	83	430	0

*Cloning were made on day 30 after initiation of the culture *in vitro*.

DISCUSSION

Tremendous advances have been made in African trypanosome cultivation systems over the past several years (Hirumi *et al.*, 1977; Hill *et al.*, 1978a, 1978b; Brun *et al.*, 1981; 1984). These reports showed that a few mammalian cell lines derived from bovine blood, Chinese hamster lung cells, embryo of *Microtus montanus*, African wild bovidae organ cells could support the continuous growth of bloodstream forms of African trypanosomes, although the functional role of feeder layers in supporting the growth has not been well understood yet. In a recent study of *T. gambiense*, Balber (1983) also showed that *T. gambiense* (Wellcome TS strain) trypomastigote forms could

be maintained in the presence of primary murine bone marrow cultures. Our previous study (1983) demonstrated that fibroblast-like cells derived from C3H/He mouse buffy coat support the growth of *T. gambiense* bloodstream forms at 37°C for three months and parasites still retained their virulence for mice.

The present study showed that malignantly transformed rat glioblast (astroglioma) cells were extremely useful for *T. gambiense* cultivation as feeder cells and could continuously support the propagation of *T. gambiense* bloodstream forms *in vitro* for more than 200 days. In the present culture system, 15-20% of parasites adhered to the surface of GA-1 cells although a majority of the populations were in suspension. Similar observations have been reported in other culture systems for African trypanosomes (Hirumi *et al.*, 1977; Hill *et al.*, 1978a, 1978b; Tanner, 1980; Brun *et al.*, 1981; Balber, 1983; Yabu *et al.*, 1983). Trypanosomes could not grow in the culture system with GA-1 cells fixed with 1% glutaraldehyde, although the attachment of parasites occurred as frequent in fixed GA-1 cells as in living ones. These findings suggest that the system without living feeder layer cells failed to maintain growth of *T. gambiense* populations and it seemed that physical contact with the feeder layer cells was essential for the propagation of African trypanosome bloodstream forms. GA-1 cells produce and secrete growth-promoting factors (range of molecular weights: 40,000-50,000 Mr) for glioblast cells into their culture medium (Kato *et al.*, 1984). GA-1 cell-extracts (40μg/ml) were also capable of stimulating DNA synthesis in quiescent normal glioblasts. However, as demonstrated here, it seemed that the secreted factors into the culture medium and the factors from cell-extracts had nothing to do with *T. gambiense* propagation.

In the culture system used here, the best growth rate was obtained in a combination with GA-1 cells and commercially available fetal calf serum. Any other sera used here as the medium supplements were unsuitable for cultivation of *T. gambiense* bloodstream forms *in vitro*. Heat-inactivated human serum had not only the incapability of supporting trypanosome growth but also had trypanocidal activity. In general, human serum is cytotoxic for Wellcome strain of *T. gambiense* adapted to mouse (Inoki, 1952; Osaki, 1959) and *T. brucei* (Laveran, 1902), although there were *T. gambiense* (Stock TH-3; Brun *et al.*, 1981) and *T. brucei* (Jenni and Brun, 1982) resistant to inactivated human serum. For these stocks, inactivated human serum as medium supplements resulted in successful growth. In the present culture system, *in vitro* cloning of cultured *T. gambiense* bloodstream forms could be successfully performed. Although there were no human serum-resistant clones selected by the cloning from sensitive populations, our system may easily allow separation of variant parasites occurring in homologous populations.

SUMMARY

Rat astroglioma cell line (GA-1) was extremely useful for long-term *in vitro* cultivation of *Trypanosoma gambiense* bloodstream forms. Parasites could be continuously grown at 37°C for more than 200 days in the culture system, consisted of HEPES-buffered RPMI 1640 (pH 7.2, 300 milliosmole/kg) supplemented with 20% inactivated fetal calf serum in the presence of GA-1 cells. Parasites cultured for more than 200 days still retained not only their virulence for mice but also their original antigenic type. Morphologically, they resembled host infected bloodstream forms by way of having a subterminal kinetoplast and surface coat. The best growth rate of trypanosomes was obtained with 1×10^6 GA-1 cells/25 cm^2

culture flask. Under this culture condition trypomastigote form populations increased in number up to 7-8 × 10^6 trypanosomes/ml by day 3 after initiation of the culture. The population doubling time in this culture system within the first 24 hours was almost the same as in mice. Most of the cultured trypanosomes were in suspension, but 15-20% of the parasites adhered to the surface of GA-1 cells. The culture system was also shown to be useful for cloning of *T. gambiense* which is important for separation of mutants.

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REFERENCES

BALBER, A. E., (1983). Primary murine bone marrow cultures support continuous growth of infectious human trypanosomes. *Science, 220* : 421.

BALBER, A. E., (1984). Growth of *Trypanosoma brucei gambiense, T. b. rhodesiense,* and *T. congolense* in bone marrow cultures of genetically anemic Sl/Sl^d mice. *J. Parasitol., 70* : 179.

BRUN, R., JENNI, L., SCHONENBERGER, M. and SCHELL, K. F., (1981). *In vitro* cultivation of bloodstream forms of *Trypanosoma brucei, T. rhodesiense,* and *T. gambiense. J. Protozool., 28* : 470.

BRUN, R., GROOTENHUIS, J.G., KUNZ, C. and SCHONENBERGER M., (1984). *In vitro* cultivation of *Trypanosoma (T.) brucei* bloodstream forms using cell lines and sera from African wild bovidae. *J. Parasitol., 70* : 836.

DOYLE, J.J., HIRUMI, H., HIRUMI, K., LUPTON, E.N. and CROSS, G.A.M., (1980). Antigenic variation in clones of animal-infective *Trypanosoma brucei* derived and maintained *in vitro. Parasitology, 80* : 359.

HILL, G.C., SHIMER, S., CAUGHEY, B. and SAUER, S., (1978a). Growth of infective forms of *Trypanosoma* (T.) *brucei* on buffalo lung tissue culture cells. *Acta Trop., 35* : 201.

HILL, G.C., SHIMER, S., CAUGHEY, B. and SAUER, S., (1978b). Growth of infective forms of *Trypanosoma rhodesiense in vitro,* the causative agent of African trypanosomiasis. *Science, 202* : 763.

HIRUMI, H., DOYLE, J.J. and HIRUMI, K., (1977). African trypanosomes : cultivation of animal-infective *Trypanosoma brucei in vitro. Science, 196* : 992.

HIRUMI, H., HIRUMI, K., DOYLE, J.J. and CROSS, G.A.M., (1980). *In vitro* cloning of animal-infective bloodstream forms of *Trypanosoma brucei. Parasitology, 80* : 371.

INOKI, S., (1952). A new esperimental method and genetical interpretation on the antigenic variation in *Trypanosoma gambiense. Med. J. Osaka Univ., 3 :* 81

JENNI, L. and BRUN, R., (1982) A new *in vitro* test for human serum resistance of *Trypanosoma (T.) brucei. Acta Trop., 39*: 281.

KATO, T., ITO, J., ISHIKAWA, K., MIZUTANI, K., TANAKA, R., WAKABAYASHI, S., HORIUCHI, I., KATO, K. and KANO-TANAKA, K., (1984). The absence of differential-promoting response of astroglioma cells to glia maturation factor. *Brain Research, 301* : 83.

LAVERAN, A., (1902). De l'action du serum humain sur le trypanosome du nagana *(Trypanosoma brucei). C. R. Acad. Sci., 134* : 735.

OSAKI, H., (1959). Studies on the immunological variation in *Trypanosoma gambiense* (serotypes and the mode of relapse). *Biken J.*, *2* : 113.

TAKAYANAGI, T., NAKATAKE, Y. and ENRIQUEZ, G. L., (1974). Attachment and ingestion of *Trypanosoma gambiense* to the rat macrophage by specific antiserum. *J. Parasitol.*, *60* : 336.

TANNER, M., (1980). Studies on the mechanisms supporting the continuous growth of *Trypanosoma (Trypanozoon) brucei* as bloodstream-like form *in vitro*. *Acta Trop.*, *37* : 203.

YABU, Y., TAKAYANAGI, T., SATO, S., KATO, H., SUZUKI, H., and KATO, S., (1983). *In vitro* cultivation of *T. gambiense*. *Jap. J. Parasitol.*, *32* : 63. (in Japanese)