In Vitro Cultivation of Bloodstream Forms of *Trypanosoma brucei gambiense* with Fibroblasts from Various Organs of Mouse

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Abstract: A modified system was used for the cultivation of *Trypanosoma brucei gambiense* (*T. b. gambiense*). The system consists of a feeder layer of new fibroblasts derived from different organs of ICR mouse, Eagle's Minimum Essential Medium (MEM) supplemented with 5% heat inactivated fetal bovine serum and 5% calf serum. Bloodstream forms of *T. b. gambiense* were cultured with mouse fibroblasts from various organs to simplify culture system and to clarify the effect of fibroblasts. Cultures were initiated with bloodstream forms from infected ICR mice. By day 8 a maximum density of 1.8×10^7 parasites/ ml/ day was obtained from new fibroblasts derived from muscles and brain. L-cells, established fibroblasts from muscles and new fibroblasts from heart, kidney and skin could not support the growth. These cultured parasites were infective for mice and retained the morphological characteristics of long slender bloodstream forms.

INTRODUCTION

Trypanosoma brucei gambiense is one of the causative agents of trypanosomiasis, the so called African sleeping sickness. The parasites are extracellular protozoa and mainly reside in the interstitial fluids and blood of infected vertebrates.

Continuous cultivation of these parasites have been attempted for many years, but have met with great difficulties. It was only in 1977 when Hirumi et al. (1977) had succeeded in cultivation of bloodstream forms of *Trypanosoma brucei brucei* (*T. b. brucei*) using feeder layers of bovine fibroblast-like cells derived from pulmonary fluid collected from cattle and peripheral blood from Boran Cross steer. Since then, several authors reported successful cultivation of *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense* using various mammalian cells and various sera. Hill et al. (1978) used buffalo lung and Chinese Hamster lung cells and 20% heat inactivated fetal bovine serum, Brun et al.

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(1981) used fibroblast-like cells isolated from embryos of New Zealand white rabbit, Mountain vole, Microtus montanus, human lung cells and 20% heat inactivated rabbit serum, Brun et al. (1984) isolated fibroblasts from different organs of African wild bovidae and used 20% sera from rabbit, goat, impala, human and gazelle and Andrew (1983) used a feeder layer from murine bone marrow cells and 25% horse serum.

These culture system gave a convenience for perfoming biological experiments without effect from host mammals. However, preparation of these feeder layer cells such as bovine fibroblasts, bone marrow cells, human embryo lung cells and of sera such as rabbit, human, goat and impala have some difficulties in common laboratories.

In the present experiment we attempted to simplify the culture system by using fibroblasts from mouse and fetal bovine or calf serum which are available in most laboratories, and to examine the ability of fibroblasts isolated from various organs of mouse in supporting the growth of bloodstream forms of *T. b. gambiense*.

MATERIALS AND METHODS

Parasites: All of our experiments were carried out with *T. b. gambiense*, Wellcome strain which was first given to the Research Institute for Microbial Diseases, Osaka University in 1956 by Dr. Max C. McCohen of the Eli Lilly Research Laboratories, Indianapolis, Indiana, U. S. A., and later on introduced to the Institute of Tropical Medicine, Nagasaki University.

Fibroblasts from various organs of mouse: New born mice (2 days old) were killed and brain, kidney, heart, muscles and skin were isolated into separate dishes, rinsed with MEM containing 10 U/ml heparin sodium, 100 U/ml penicillin and 100 μg/ml streptomycin and minced.

These minced tissue pieces were suspended in MEM supplemented with 10% fetal bovine serum, dispensed into tissue culture dishes (Falcon 100 x 20mm) and incubated at 37°C in 5% CO_2 incubator. Fibroblasts developed from attached tissue pieces and extended to surrounding area within 2-3 days of incubation, thereafter unattached pieces were removed by changing medium. Fibroblasts were then maintained by changing medium after every 3 days and transferring a quarter of cells into a new dish once a week and used for experiment within three months after the initial cultivation. Established fibroblasts from mouse muscles which showed slight morphological change and more rapid growth about five months after the first culture, and L-cells were used for comparison.

Cultivation of *T. b. gambiense* with various fibroblasts: When parasitaemia of infected mice (ICR, more than 8 weeks old) was high enough (about 10^8 parasites/ml), the mice were sacrificed and blood was collected by cardiac puncture. Trypanosomes were separated from host blood components by centrifugation and chromatography methods (Lanham and

Godfrey, 1970). The parasites were washed once by centrifugation, suspended in MEM containing a mixture of 5% fetal bovine serum and 5% calf serum, counted and thereafter 3×10^5 trypanosomes were inoculated into each tissue culture dish with confluent monolayer cells of fibroblasts from different organs. The culture dishes were incubated at 37°C in 5% CO_2 incubator.

Culture medium used for propagation of the trypanosomes was MEM with Earle's salts (GIBCO), containing penicillin 200 U/ml, streptomycin 100 μg/ml and pH regulated to 7.4. Before use in one part of the experiment the medium was supplemented with 10% calf serum and in another experiment a mixture of 5% calf serum and 5% heat inactivated fetal bovine serum was added.

Initiation of the cultures was carried out either by centrifuging the supernatant medium and return all the pelleted trypomastigotes into culture dishes or by replacing half of the old medium by fresh one. Either procedure was repeated until trypanosomes showed propagation or completely disappeared from the cultures. In those cultures where growth of trypanosomes became established in the feeder layers, whole medium was changed once every day. The cultures were daily examined with inverted phase contrast microscope and trypanosomes were counted from the culture supernatant fluid.

Morphological observation and infection to mice: In order to check morphology of cultured trypanosomes, medium from the culture dishes was centrifuged at 1,800 *g* for 10 min, the supernatant fluid was discarded and smears were prepared from the pellet. The smears were fixed with methanol for 10 min, Giemsa-stained for 30 min and then observed under light microscope. To determine the infectivity and pathogenicity of trypanosomes after 60 days of *in vitro* cultivation, 1×10^3 parasites from culture were inoculated into each of five ICR mice. The same density of parasites from infected mouse blood was also used to inoculate the same number of ICR mice.

RESULTS

Growth of *T. b. gambiense* in different feeder layers: Trypanosomes were observed to penetrate and move actively between the fibroblasts of feeder layers derived from muscles and brain within 6 h after inoculation, but scarcely in fibroblasts from other sources. In the former fibroblasts, the number of intercellular trypanosomes gradually increased and after 3 – 4 days they formed clusters (Photo. 1.). Successful growth of trypanosomes was obtained in fibroblast cultures from brain and muscles within one week after transfer from infected mice, but not in other fibroblast cultures (Fig. 1.).

Stable propagation of trypanosomes was maintained through experimental period (8 weeks), and a final density 2×10^7 parasites/ ml was continuously obtained every day (Fig. 2.). Better growth of trypanosomes was obtained when medium supplemented with a mixture of 5% fetal bovine serum and 5% calf serum was used, and the cultured

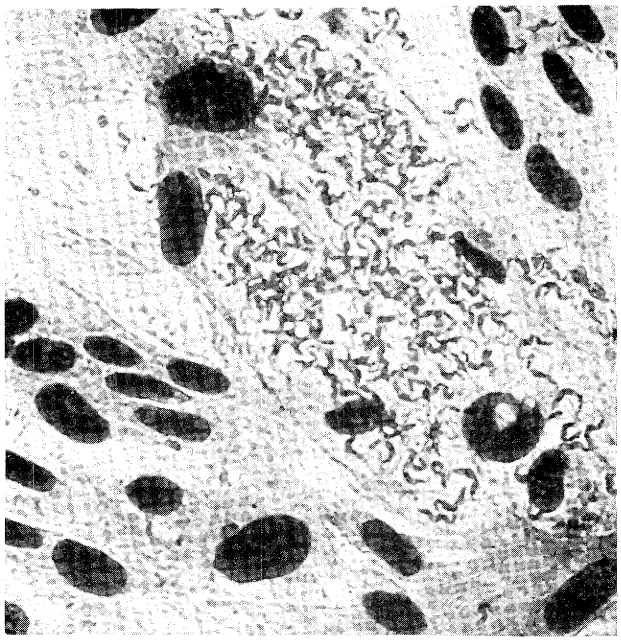

Photo. 1. *T. b. gambiense* forming clusters between the feeder layer cells of fibroblasts from mouse brain. The feeder layer was gently washed with saline, dried, fixed with methanol for 15 min and Giemsa-stained for 45 min.

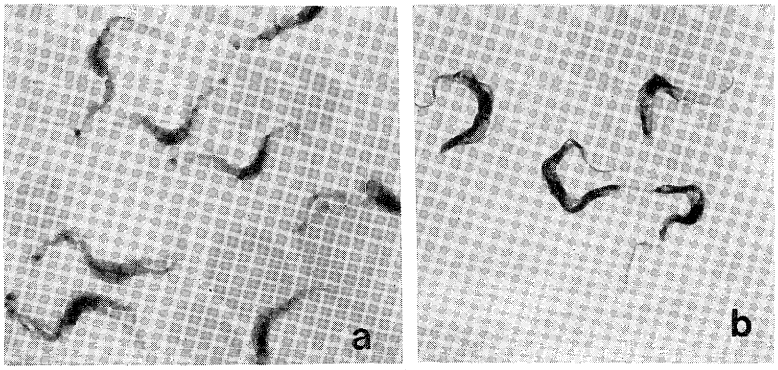

Photo. 2. Smear of *T. b. gambiense* from mouse blood (a), and from culture after 60 days of cultivation (b).

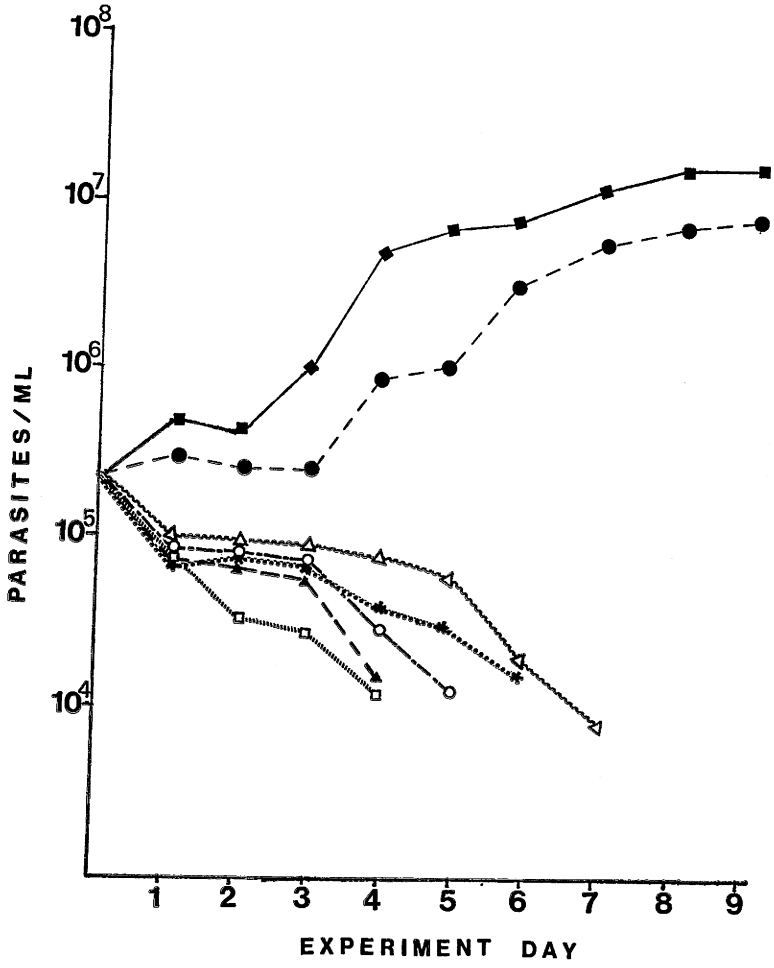

Fig. 1. Initial growth of trypanosomes in various fibroblast cultures after transfer from mice infected with *T. b. gambiense*. Bloodstream forms of the parasites (3×10^5) were inoculated into each of the culture dishes (Falcon 100×20mm) with confluent fibroblasts from different sources. After 24h incubation they were collected by centrifugation at 1,800 *g* for 10 min, counted and again introduced into the same dishes with fresh medium (MEM supplemented with 5% calf serum and 5% fetal bovine serum). This procedure was repeated until parasites showed propagation or disappearance. After the 4th day when trypanosomes showed stable propagation in fibroblast cultures derived from brain and muscles, complete exchange of medium was done every 24h in those cultures.

Kidney Fibroblast
L-cell
Skin Fibroblast
Heart Fibroblast
Brain Fibroblast
Muscles Fibroblast
Estab. muscles Fibroblast

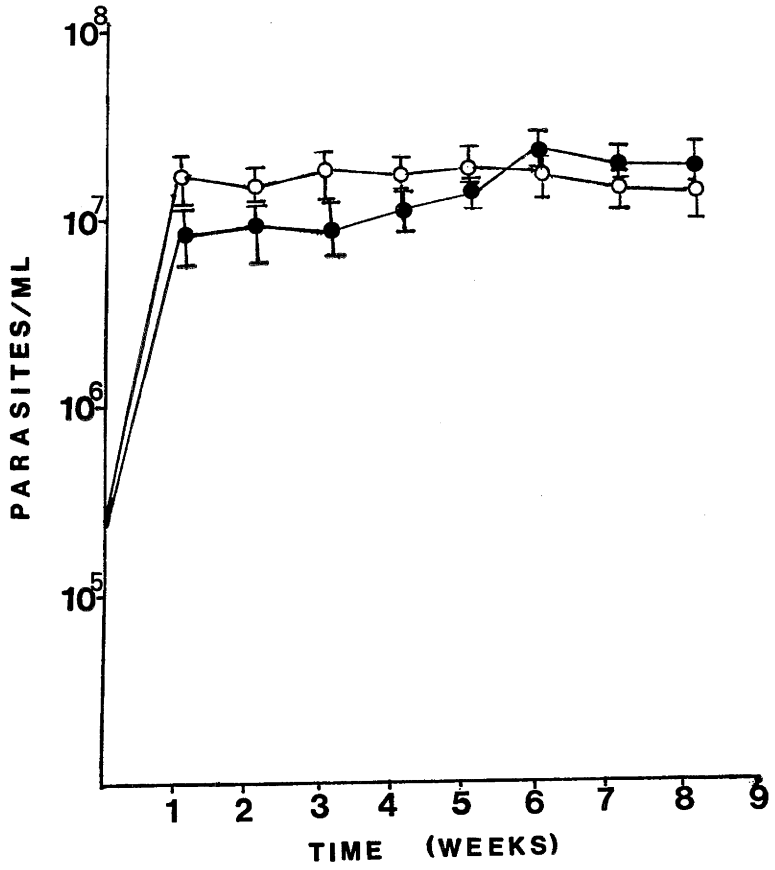

Fig. 2. Trypanosome growth in fibroblast cultures from brain and muscles after the initial establishment of trypanosome growth shown in Fig. 1. Counting free parasites in overlaid medium and medium change were done every day. The mean number counted at interval of one week was shown here.

○—○ Brain Fibroblast

●—● Muscle Fibroblast

trypanosomes were morphologically indistinguishable from bloodstream forms from mice (Photo. 2.). On the contrary when MEM supplemented with 10% calf serum was used, the growth of trypanosomes continued for two weeks, after which the cultured trypanosomes became sluggish, short, developed numerous eosinophilic granules in their cytoplasm, lost their flagella and finally died.

Infectivity test for mice: Two groups of mice which were infected either with cultured trypanosomes or trypanosomes from infected mouse blood developed infection, reached maximum parasitaemia 10^9/ ml and died after 5 days.

DISCUSSION

Up to the present moment, it is true that mammalian feeder layer cells are essential for the continuous cultivation of bloodstream forms of *Trypanosoma brucei* (Hirumi *et al.*, 1977; Hill *et al.*, 1978). It is well accepted that penetration and formation of clusters between cells of the fibroblast type are much better than epithelial cells (Brun *et al.*, 1984; Agda *et al.*, 1985).

In this study we cross-checked the ability of fibroblasts from different organs of the mouse to support the growth of *T. b. gambiense*. Fibroblasts isolated from brain and muscles were capable of supporting the growth of *T. b. gambiense* and the maximum number of parasites obtained per day was 2×10^7/ ml. This number was equivalent to those reported by other authors (Brun and Jenni, 1977; Brun and Schönenberger, 1979; Gray *et al.*, 1981; Cunningham *et al.*, 1981). Futhermore, the penetration and formation of clusters between the feeder layer cells were much better in new fibroblasts from brain and muscles than fibroblasts from other organs. This close association between fibroblasts and intercellularly localized trypanosomes might have influenced the growth of trypanosomes. However, whether these fibroblasts produced trypanosomal growth supporting factors which stimulated intercellular trypanosomes are not yet known. Our investigations are in agreement with Tanner's report (Tanner, 1980), who found out that close-interaction between trypanosomes and fibroblasts was necessary for the continuous growth of *Trypanosoma brucei*. Moreover, our result showed that, the effect of fibroblasts to support trypanosome growth was different according to the derived organs, even though they were from the same litter of ICR mice.

It is said that conditioned fibroblast culture to support trypanosome growth is related to the growth rate of fibroblasts (personal cummunication). However, growth rates of these fibroblasts from different organs were examined but did not show much difference.

Throughout our investigation two types of sera were used as medium supplements. 10% calf serum could support the growth of trypanosomes in fibroblasts from brain and muscles for two weeks only, but it could not support the growth of trypanosomes in other fibroblasts for more than a week. A mixture of 5% fetal bovine serum and 5% calf

serum gave excellent continuous growth of trypanosomes in fibroblasts from brain and muscles comparable to 10% fetal bovine serum, but could hardly support the growth of trypanosomes in fibroblasts from heart, kidney, skin, established fibroblasts from muscles and L-cells. We have not yet established whether these sera supplied growth factors or they just stimulated the fibroblasts which in turn produced the trypanosomal growth stimulating effect.

In the present experiments it is difficult to make a definite conclusion on the different ability of these fibroblasts in supporting the growth of trypanosomes. Nevertheless, by comparing fibroblasts which can support the growth of trypanosomes with those which cannot, trypanosomal growth factors may be analysed more exactly.

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マウスの異った臓器由来の線維芽細胞を用いた *Trypanosoma brucei gambiense* 血流型原虫の試験管内培養

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ガンビアトリパノソーマの血流型の簡便な培養法を得るために，マウスの異った臓器由来の線維芽細胞を共生させ，子牛血清・牛胎児血清を混じた MEM 培地を使用して実験を試みた．結果感染マウスより移植した血流型原虫は脳および筋肉由来の線維芽細胞共生条件で活発に細胞間隙に侵入し，3－4日後にはそこで増殖した原虫集塊が形成されるのが認められた．その後この両線維芽細胞の系では原虫は活発に増殖し，連日全部の培養上清を交換することにより，毎日 2×10^7/ml の濃度で培養液中に原虫を得ることができた．この原虫は現在9週以上にわたって継代されているがその感染性は血流型と同じに保たれていることから，昆虫内増殖型への形態変移は行われていないことが判る．この増殖効果は心臓・腎臓・皮膚由来の線維芽細胞，筋肉由来だがすでに株化した線維芽細胞，L-cell などには認められなかった．また10％子牛血清のみでの原虫の増殖は短期間しか認められず牛胎児血清が必須であったが5％牛胎児血清，5％子牛血清を昆じた MEM 培地を利用することにより10％牛胎児血清を用いるのと同様な増殖が得られた．