# African Trypanosomes: Cultivation of Animal-Infective *Trypanosoma brucei* in vitro

Abstract. Trypanosoma brucei *grew in the presence of bovine fibroblast-like cells in Hepes-buffered RPMI 1640 medium with 20 percent fetal bovine serum for more than 220 days at 37°C. The organisms grown in this system were infective to mammalian hosts, retained the morphological and biochemical characteristics of long slender bloodstream forms, and displayed variant-antigen on their surfaces.*

In previous attempts to cultivate African trypanosomes in vitro, the bloodstream forms of *Trypanosoma brucei* rarely retained their characteristic morphology and infectivity for mammalian hosts (*1, 2*); in such systems bloodstream *T. brucei* rapidly transformed into insect midgut forms (*1–3*). These cultures were maintained continuously only at temperatures near 25°C. The lack of an adequate system for cultivating animal-infective salivarian trypanosomes in vitro has hindered the fundamental studies of African trypanosomiasis and the development of vaccine and chemotherapeutic technology against this disease. We have now developed a system that supports the long-term propagation of animal-infective *T. brucei* in vitro.

Trypanosomes (*T. brucei*, strain 427) were obtained from a lethally irradiated mouse (C57BL/10ScSn, hereinafter referred to as C57BL, obtained from Bantin & Kingman, Grimston, U.K.) that was showing a rising parasitemia. The parasites were separated from the blood by density centrifugation at 350*g* for 15 minutes (Lymphoprep density 1.077 g/ml). Trypanosomes removed from the interface layer were washed three times with Hanks balanced salt solution (BSS) by sequential centrifugation at 1400*g* (maximum) for 5 minutes at 4°C. The isolated organisms were resuspended in BSS to a final concentration $2 \times 10^4$ trypanosomes per milliliter, and this suspension was used as the inoculum.

Pulmonary fluid was collected from cattle infected with *Theileria parva* (Muguga strain) shortly after they were killed. This fluid was used to initiate cultures of fibroblast-like cells in 25 $cm^2$ Falcon plastic culture flasks (T-25) with RPMI 1640 medium (*4*) containing 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 20 percent heat-inactivated fetal bovine serum (FBS) at 37°C. Confluent fibroblast-like cell monolayers were subcultured at 5- to 7-day intervals. This cell line (*5*), designated ILR-BPF-376, was used during the early stages of the experiment. Examination of Giemsa-stained ILR-BPF-376 cells with the light microscope, and failure of the cells to produce theileriosis upon inoculation into susceptible cattle, indicated that the cell line was most probably free of *Theileria* parasites. Another fibroblast-like cell line, ILR-BHF-476, was prepared from peripheral blood from a healthy yearling Boran cross steer (*6*). This cell line, which differed morphologically from ILR-BPF-376 cell line and formed multiple cell layers, was used later in the experiment.

We attempted to grow *T. brucei* under 96 different culture conditions (12 different media, four different concentrations of FBS, and in the presence or absence of ILR-BPF-376 cells) using Disposo-Trays with 13-mm diameter wells (Linbro Chemical). Each medium (*7*) was supplemented with 5, 10, 20, or 30 percent heat-inactivated FBS (ABS). Each well containing ILR-BPF-376 cell monolayers (3 days old) was washed three times with BSS, and then 0.9 ml of the medium to be tested was added. Also, wells were prepared in the same manner, but without ILR-BPF-376 cells. Duplicates were made for each culture system. Each well then received 0.1 ml of the original inoculum containing $2 \times 10^3$ trypanosomes per well. The trays were sealed and incubated at 37°C, and the cultures were examined by phase microscopy at 24-hour intervals up to day 8 of cultivation.

The trypanosomes in all wells containing cell-free media became sluggish and did not significantly increase in number during the first 24 hours of culture. All organisms were dead after 48 hours. In contrast, the trypanosomes in many wells containing ILR-BPF-376 cells increased in number and were still very motile after 24 hours of culture. The media which supported the initial growth were Eagle's basal medium with 10, 20, and 30 percent FBS; Medium 199 with 5, 10, 20, and 30 percent FBS; and RPMI 1640 medium with 20 and 30 percent FBS. In two wells which contained the RPMI 1640 medium with 20 percent FBS, the trypanosomes increased in number to $3.5 \times 10^5$/ml 96 hours after inoculation. This rate of multiplication was not achieved in any other medium supporting the initial growth. One such culture was transferred on day 4 to a freshly prepared ILR-BPF-376 cell culture in a T-25 flask containing 4 ml of the RPMI 1640 medium with 20 percent FBS. The other culture was used for the first infectivity test on day 5. In the other culture systems, trypanosomes became sluggish after 96 hours and gradually died out by day 8.

The *T. brucei* transferred to the T-25 flask continued to multiply and increased in number to $5.2 \times 10^5$ per milliliter by day 4 after inoculation. At the third passage, subcultures were made in four T-25 flasks (two with and two without ILR-BPF-376 cells). Each flask received 0.5 ml of the previous culture fluid and 4.5 ml of the fresh medium, and was incubated at 37°C. No multiplication was evident in the cell-free medium. On the other hand, the organisms multiplied in the presence of the fibroblast-like cells and increased in number to $2.8 \times 10^5$ trypanosomes per milliliter on day 5 of culture. This confirmed that the presence of host cells was essential to support the propagation in vitro of animal-infective bloodstream trypomastigotes of *T. brucei* at 37°C. We therefore made further serial subcultivations at this temperature using the RPMI 1640 medium (*p*H 7.2 and 290 milliosmole/kg) in either ILR-BPF-376 (up to passage 14) or ILR-BHF-476 (from passage 15 to date) cell cultures. During the past 8 months, we have made a total of 1246 subcultures in either T-25 culture flasks or T-75 (6 ml or 18 ml of medium per flask, respectively), initiating the cultures with various concentrations (10 to $10^4$ trypanosomes per milliliter) of cultured *T. brucei*. The organisms still continue to grow under these conditions and are now at passage 181 (223 days in vitro).

In cultures containing compact ILR-BPF-376 cell monolayers, the trypanosomes swam along the surface of the monolayers, often attaching to the cell membranes for a short time. On the other hand, in cultures containing loosely formed multiple layers of ILR-BHF-476 cells, many long slender forms appeared in intercellular spaces of cell sheets. The spaces were often elongated and of various sizes, and contained densely accumulated long slender forms of *T. brucei* (Fig. 1A). Such foci were more abundant in older cultures. Many free-swimming trypanosomes were also present in ILR-BHF-476 cultures (Fig. 1B). No cytopathic effects were distinguishable in either ILR-BPF-376 or ILR-BHF-476 cells by phase microscopy.

The morphology of trypanosomes maintained by rapid passage (at 24 hour intervals) in cultures was identical to that of the long slender forms of *T. brucei* in the bloodstream in vivo, as judged by air-dried, methanol-fixed smears stained by the May and Grundewald–Giemsa method (Fig. 2A). In cultures where parasite numbers were diminishing following the

period of maximum growth, the morphology of the trypanosomes was markedly altered: they became shortened and frequently had numerous eosinophilic granules in their cytoplasm. Bizarre multinucleate and giant forms were also present. All forms of cultured trypanosomes had subterminal kinetoplasts. Growth patterns and numbers of these polymorphic forms varied considerably depending on the number of trypanosomes inoculated, the predominant forms in the inoculum, and age of cultures. Successful subcultivations were usually obtained at 24-hour intervals from the previous cultures which contained predominantly long slender forms ($2 \times 10^5$ to $1 \times 10^6$ trypanosomes per milliliter). The subcultivations were made by dividing the previous culture fluid into three parts and transferring each part to a new flask which contained the bovine fibroblast-like cells and fresh culture medium (one part of the previous culture fluid and two parts of the fresh medium). Organisms subcultured in this way generally multiplied three- to fivefold with a population doubling time of 10 to 12 hours during 24 hours of culture. Under optimum conditions, trypanosomes increased in number 16-fold within 24 hours (population doubling time, 6 hours). In such cultures, most of the organisms were very motile long slender forms. Numerous dividing forms were also present. When cultures were maintained for more than 3 days without changing the medium, trypanosomes rapidly transformed to short forms. Subcultures prepared from such cultures took from 4 to 12 days to recover, depending on the number of long slender forms present in the inoculum. Organisms in subcultures prepared from the inoculum in which long slender forms were not detected gradually died out, indicating that this transformation under such culture conditions was irreversible.

The presence of mitochondrial enzyme (lipoyl dehydrogenase), as demonstrated by the "diaphorase" technique, is characteristic of the intermediate and stumpy bloodstream forms of *T. brucei* and of the noninfective trypomastigote forms previously grown in vitro, but the enzyme is not present in the long slender bloodstream forms in vivo (*8, 9*). The presence of this enzyme could not be demonstrated in the mitochondria of long slender trypanosomes grown in vitro under the conditions reported here, although the enzyme was clearly demonstrable in the mitochondria of control bloodstream *T. congolense* (*10*). Some short forms found in cultures maintained for more than 3 days without the medium being changed resembled morphologically the intermediate and stumpy forms of bloodstream *T. brucei*. In contrast to the intermediate and stumpy bloodstream forms in vivo, however, the presence of lipoyl dehydrogenase could not be demonstrated in the mitochondria of the short forms of cultured trypanosomes.

In previous culture systems in vitro, the transformation to noninfective trypomastigote forms was accompanied by a loss of the surface "coat" carrying the variant specific antigens of bloodstream *T. brucei* (*11*). The surface coat varied in thickness according to the preparative techniques used for electron microscopy (*12*). The size of the mitochondria also markedly increased in the course of the transformation in vivo (*8, 13*). In the present culture system, the ultrastructure of the mitochondria and surface membrane of cultured *T. brucei* (day 38 in vitro, passage 9) (Fig. 2B) resembled those of the bloodstream trypanosomes obtained from infected mice as examined by a standard electron microscopy technique (*14*).

The presence of variant antigen on the surface of living bloodstream forms of *T. brucei* has conventionally been demonstrated by agglutination testing with antiserums obtained from infected animals bled after an appropriate period (*15*). Recently, the surface antigen responsible for variant specificity has been isolated and characterized as a glycoprotein (*16*). By means of direct and indirect immunofluorescence techniques similar to those described for *Leishmania* (*17*), it has been shown with isolated living bloodstream trypanosomes that antiserums prepared against purified variant specific glycoproteins as well as antiserum obtained from infected animals react specifically with the surface variant antigens of the trypanosomes from which they were derived (*16, 18*).

The presence of variant antigen on the surface of trypanosomes cultured in the

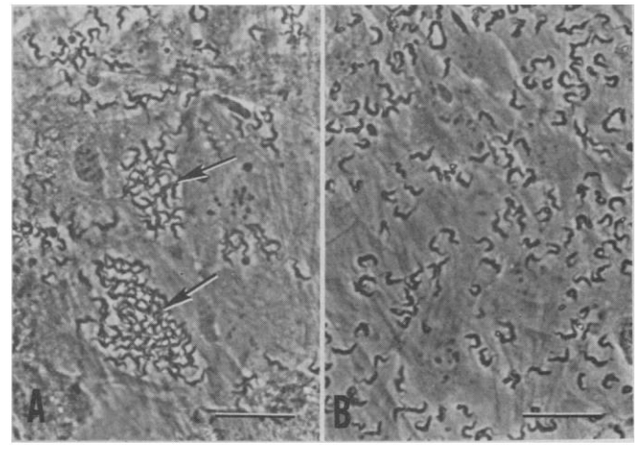


Fig. 1. *Trypanosoma brucei* grown in the presence of bovine fibroblast-like cells (ILR-BHF-476). The trypanosomes are at passage 59 (24 hours old) and have been cultured for a total 103 days in vitro. (A) Intercellular accumulations of long slender forms (arrows); (B) free-swimming organisms. Fixed with glutaraldehyde. Phase contrast micrographs. Scale bar, 50 $\mu$m.

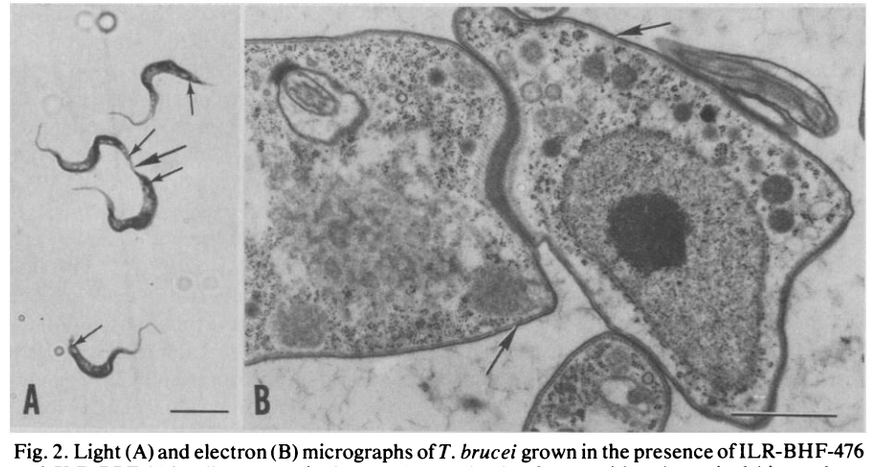


Fig. 2. Light (A) and electron (B) micrographs of *T. brucei* grown in the presence of ILR-BHF-476 and ILR-BPF-376 cells, respectively. (A) Long slender forms with subterminal kinetoplasts (small arrows), morphologically similar to long slender bloodstream *T. brucei*. Large arrow denotes a dividing form. The trypanosomes are at passage 34 (24 hours old) and have been cultured for a total 78 days in vitro. May and Grundewald–Giesma stain. Scale bar, 10 $\mu$m. (B) Thin section of long slender forms bounded by high electron-opaque plasma membranes (arrows). Note the absence of "well developed" mitochondria. Fixed with glutaraldehyde and osmium tetroxide, and stained with uranyl magnesium acetate followed by lead citrate. The trypanosomes are at passage 9 (48 hours old) and have been cultured for a total 38 days in vitro. Scale bar, 5 $\mu$m.

presence of the bovine fibroblast-like cells at 37°C was demonstrated directly by agglutination and immunofluorescence, with both an antiserum raised to the variant specific glycoprotein of the predominant variant population in the strain and an antiserum recognizing all the variant types present in this population. The latter antiserum was obtained by infecting a rabbit with $10^6$ trypanosomes taken after 50 days of culture and bleeding the rabbit 6 days later. Trypanosomes were tested for the presence of variant antigen after 8, 20, 49, and 68 days of culture in vitro at 37°C. Bloodstream trypanosomes of the original isolate used to initiate the culture were used as controls in these experiments.

On days 5, 8, and 20 after initiation of the culture in vitro, lethally irradiated C57BL mice were inoculated intraperitoneally with $10^5$ cultured trypanosomes. All the mice developed lethal infections. On days 29, 49, 63, 98, 102, 107, 183, 192, 193, 197, 201, 203, 207, 210, and 213 infectivity tests (*19*) were carried out by inoculating groups of normal C57BL mice intraperitoneally with tenfold dilutions of suspensions of cultured trypanosomes, containing $10^6$ to 10 such organisms. All mice inoculated with cultured trypanosomes consistently developed lethal infections in such experiments. On day 29 of culture, two yearling cattle were also inoculated intravenously with $1.8 \times 10^7$ and $4.2 \times 10^6$ cultured trypanosomes, respectively. Patent infections in these cattle were detected 5 days after inoculation. In cultures (passage 3, 7 days old, after a total of 15 days in vitro) where long slender forms were not detected, trypanosomes which altered to short forms did not infect normal C57BL mice.

The ability of the normal bovine fibroblast-like cells to support the initial cultivation in vitro of bloodstream forms of *T. brucei* (strain 427) was also investigated. Freshly isolated bloodstream forms were resuspended in RPMI 1640 medium ($10^5$ trypanosomes per milliliter), placed in eight T-25 flasks (6 ml per flask) containing ILR-BHF-476 cell layers, and incubated at 37°C. At 24-hour intervals during the initial 7 days of culture half of the culture fluid (3 ml per flask) was replaced with fresh medium, resulting in a daily reduction by half of the trypanosome population. On day 7 the average concentration of the organisms had reached $3.3 \times 10^5$ per milliliter, and successful subcultivations were made from all original cultures by dividing them into three parts in the same manner as described above. On days 9, 14, 25, and 55 after initiation of the culture, groups of normal C57BL mice were inoculated intraperitoneally with $10^5$ cultured long slender bloodstream forms of *T. brucei*. All mice developed patent infections within 3 days. This indicated that long-term propagation of bloodstream *T. brucei* in vitro can also be initiated with healthy bovine fibroblast-like cells.

Previous attempts to culture bloodstream forms of *T. brucei* in the presence of mouse L-cell monolayers at either 27° or 37°C were unsuccessful (*2*). In such cultures, the organisms lost their ability to infect mammalian hosts after a maximum of 7 days of cultivation in vitro.

We also investigated the ability of such mouse L-cell monolayers to support the initiation and establishment of cultures of bloodstream trypanosomes in vitro. Cultures were initiated in the presence of L-cell monolayers in the same manner as described for the ILR-BHF-476 cell system. In such cultures, however, the bloodstream forms took 14 days to reach a population density from which successful subcultivations were possible. These cultured trypanosomes were morphologically identical to those grown over the bovine fibroblast-like cells and retained their infectivity for mice when tested after 20 and 50 days of culture. Extreme care was necessary in the handling of the initial cultures as regards both the amount and timing of medium replacement. This was decided daily after assessing the number of trypanosomes and their viability in each culture. On the contrary, trypanosomes maintained for 90 days at 37°C in bovine fibroblast-like cell cultures continued to grow without difficulty when transferred to mouse L-cell cultures, and they retained their infectivity for mice until such cultures were terminated 55 days later. This indicates that the mouse L-cell system is inferior to the bovine fibroblast-like cell system in its ability to support the initial cultivation of bloodstream *T. brucei*, but it is adequate to maintain organisms previously adapted to the conditions of cultivation in vitro.

The results reported here demonstrate that bloodstream forms of *T. brucei* can be propagated in vitro at 37°C, and that these forms retain in vitro their specific surface antigens, and their morphological and biochemical characteristics, as well as their ability to infect animals. The system we describe may allow elucidation of the physiology, biochemistry, and mechanisms of antigenic variation of this form of the parasite, and facilitate the development and testing of new trypanocidal drugs.

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**References and Notes**

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6. The buffy coat cells obtained from 20 ml of fresh bovine blood were resuspended in 20 ml of RPMI 1640 medium containing 20 percent fetal bovine serum, placed in a Falcon plastic culture flask (T-75) and incubated at 37°C. After 18 hours of cultivation, the culture fluid was transferred to a new T-75 culture flask so that most of the macrophages which adhered to the original culture flask were removed. Fibroblast-like cells, lymphocytes, and some macrophages adhered to the new culture flask and increased in number during the next 14 days, the fibroblast-like cells tending to attach more firmly to the surface of the culture flask than the others. Cells other than fibroblast-like cells were removed by repeated trypsinization (0.25 percent trypsin, ABS) of the culture during this period. During trypsinization the culture was periodically observed with an inverted phase microscope, and when the other cell types detached from the flask the trypsin solution was replaced with the fresh medium. When cell layers consisted predominantly of fibroblast-like cells, they were released from the culture flask by means of a trypsin-EDTA mixture (ABS) and subcultured in the conventional manner. A homologous fibroblast-like cell population (ILR-BHF-476 cell line) was obtained after four subsequent serial cultivations. At present the cell line is at passage 17.
7. The media tested were Chees FOMI (ABS); CMRL 1066 medium (Gibco); Eagle's basal medium with 25 m*M* Hepes (Gibco); Eagle's minimum essential medium for monolayers (Gibco); Fisher's medium (ABS); lactalbumin hydrolyzate medium with Hanks BSS (Gibco); Leibovitz L-15 medium (Gibco); McCoy's 5a medium, modified (Gibco); Medium 199 with Hanks BSS (Gibco); Minowada's medium (ABS); Mosquito medium (Gibco); and RPMI 1640 medium with 25 m*M* Hepes (ABS).
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