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In vitro cultivation of animal-infective forms of a West African *Trypanosoma vivax* stock

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Summary

Animal-infective forms of a West African *Trypanosoma vivax* stock were grown in culture for three months using Minimum Essential Medium (MEM) with Earle's salts, supplemented with 20% inactivated goat serum over fibroblast-like cell lines isolated from the embryo of *Microtus montanus* or of an East African Galla crossbred goat at 36.5 °C and in 4% CO₂ – 96% air. The blood-stream trypanosomes used to initiate the culture had been isolated from an infected goat. The cultured organisms grown in this system could be subcultured, were infective for mammalian hosts, retained their morphological characteristics and virulence, and could be readily established in *Glossina morsitans centralis* from goats injected with the cultured *T. vivax*.

Key words: *Trypanosoma vivax*; in vitro cultivation; animal-infective forms; *Glossina* infection.

Introduction

Trypanosoma vivax causes disease in livestock and is therefore of considerable economic importance in Africa and South America. Whereas the in vitro cultivation of *T. brucei* (Hirumi et al., 1977; Hill et al., 1978; Brun et al., 1981) and *T. congolense* (Gray et al., 1979, 1981; Hirumi et al., 1981) have recently been achieved, attempts to cultivate animal-infective *T. vivax* have been relatively unsuccessful (Trager, 1959, 1975; Isoun and Isoun, 1974). Small laboratory animals are normally refractory to infection with this trypanosome species. Hence, very little fundamental research has been conducted on *T. vivax* in contrast to the above pathogenic trypanosome species.

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Leeflang et al. (1976) isolated stocks of *T. vivax* from cattle in Nigeria, which can readily infect small laboratory animals. These parasites can be cyclically transmitted by *Glossina* (Moloo, 1981a). In this paper we report a culture system that supports the growth of animal-infective bloodstream forms of a West African stock of *T. vivax* in vitro.

Materials and Methods

Hosts

The animals used to infect and maintain tsetse flies were East African Galla crossbred goats. For infectivity tests, the goats as well as A/J mice were used.

Trypanosome stock

Trypanosoma vivax (ILRAD 417) is a derivative of Zaria Y486 which was isolated from a Zebu in Nigeria in 1973 (Leeflang et al., 1976). The history of this derivative has been described elsewhere (Moloo, 1981b).

Culture system

The culture system consisted of a mammalian cell feeder layer in Minimum Essential Medium (MEM) with Earle's salts supplemented with 20% inactivated goat serum since this breed of goat is highly susceptible to the *T. vivax* stock (Moloo, 1981a). Two cell lines were used as feeder layers: MEF, a fibroblast-like cell line isolated from whole embryos of *Microtus montanus* (Brun et al., 1981) and GEL, a fibroblast-like cell line isolated from embryonic lung tissue of the above breed of goat according to the method used for the *Microtus* cell line. The feeder layer stock cultures were maintained in MEM with Earle's salts supplemented with 10% inactivated foetal bovine serum (FBS).

For the cultivation of the trypanosomes in co-cultivation with the feeder layer cells a modified MEM was used:

450 ml	MEM with Earle's salts, without L-glutamine (GIBCO No. 320-1090)
45 ml	double-distilled water
5 ml	MEM Non-essential amino acid concentrate (100×)
3.58 g	HEPES buffer
1.0 g	Glucose
0.15 g	L-glutamine

The pH was adjusted to 7.5 with 4N NaOH. The medium was filter sterilized (0.22 µm) and stored at 4°C for up to one month.

The medium was supplemented with 20% heat-inactivated goat serum prepared from blood of East African adult Galla crossbred goats. Commercially available goat serum or FBS could not replace our own prepared serum.

All experiments were carried out in 24-well tissue culture clusters (Costar No. 3524; Costar, Cambridge, Massachusetts) using 1.0 ml serum containing medium per well. The plates were incubated at 36.5°C in 4% CO₂ – 96% air.

Isolation of trypanosomes from goat blood

A goat was injected intramuscularly with the stabilate diluted in phosphate-buffered saline-glucose (PSG). To determine the degree of parasitaemia, the animal was bled from the ear daily, and a wet blood film was examined microscopically. Citrated blood (final concentration 1%) was taken from the jugular vein of the infected goat and mixed with an equal volume of Hanks balanced salt solution (HBSS). Five ml aliquots of this suspension were put in plastic centrifuge tubes and

spun for 10 min at 1000 g at room temperature. By this centrifugation the trypanosomes were concentrated on top of the blood cells. The trypanosomes could be brought into the supernatant fluid by gently rotating the tubes by hand. The supernate was then centrifuged for 7 min at 1000 g, the pellet resuspended in the medium and the organisms counted in a Neubauer haemocytometer.

Preparation of the cultures

Wells containing almost confluent MEF or GEL feeder layer cells received fresh medium supplemented with 20% inactivated goat serum and gentamycin (10 µg/ml). Bloodstream forms were added to the wells giving a final concentration of 3×10^4 to 1.5×10^5 per ml. The cultures were regularly examined using an inverted phase-contrast microscope. The medium was partially replaced (50–90%) every day according to the trypanosome density. The same feeder layer could be used for several weeks.

Infectivity check

Infectivity of the trypanosomes in culture for mammalian hosts was tested in the mice and the goats. The mice were injected intraperitoneally with 0.2 ml culture supernate (approximately 3×10^4 to 10^5 trypanosomes) and examined daily. The goats were injected intramuscularly with 0.2 ml culture supernate and their blood examined daily.

Electron microscopy

Trypanosomes from culture and from an infected goat were centrifuged for 7 min at 1000 g and the pellets resuspended in a small volume of 2% BSA in MEM. This suspension was filled into capillary tubes, one end was sealed with plasticine and centrifuged for 10 min at 1000 g. The resulting pellets were fixed for 1½ h in 2.5% glutaraldehyde – 0.1 M cacodylate buffer (pH = 7.3) at 4° C. They were then left overnight in cold 0.2 M cacodylate buffer with 5% sucrose, postfixed for 2 h in 2% OsO₄ – 0.2 M cacodylate buffer at 4° C. The samples were block stained in 70% acetone containing 2% uranylacetate, dehydrated in acetone and propyleneoxide, and embedded in epon.

Results

Bloodstream forms of *T. vivax* could be grown in vitro in co-cultivation with 2 mammalian fibroblast-like cell lines: MEF, isolated from *Microtus montanus* and GEL, isolated from a goat embryo. With MEF bloodstream forms were cultivated for over 3 months and with GEL for over 2 months.

Only trypomastigote forms could be observed in the cultures. They were either loosely attached to the feeder cells or free in the supernatant medium. Intercellular forms between the fibroblasts could not be seen. After initiation of the cultures, about 50% of the medium was replaced after one day by carefully removing fluid from the top of the wells and replacing it with fresh medium. When the trypanosome density increased, 70–90% of the medium was exchanged daily. The attached trypanosomes were not removed by this procedure and acted as 'inoculum' for the following 24 h.

The highest trypanosome density found in wells was approximately 10^6 organisms/ml. The number of trypomastigotes removed with the daily medium replacement varied considerably between 10^5 and 7×10^5 per ml. It should be mentioned, however, that we observed distinct fluctuations in the trypanosome densities.

Subcultures onto fresh feeder layers were done approximately every 3 weeks by inoculating almost confluent MEF or GEL cultures with trypanosomes from the supernate of an established culture. Infectivity of the cultivated trypomastigotes was tested in mice about every 3 weeks and in 2 goats. The mice injected with culture forms always developed parasitaemia which was detectable on day 2 or 3. Goat 1 received trypanosomes which had been growing for 35 days over MEF. Parasitaemia was detected on day 6, and the animal died on day 20 with peak of parasitaemia. On day 7 post-injection, 200 teneral *Glossina morsitans centralis* were fed on this goat for 21 days, and then the dissected labra and hypopharynges were examined for parasites. The infection rate of these tsetse flies was 89.6% and they were able to transmit the infection to 2 clean goats which showed parasites in their blood on day 10 and 8, and they died on day 17 and 13 respectively, at peak parasitaemia. Goat 2 was injected with trypanosomes which had been growing for 59 days over GEL. The parasitaemia was first detected on day 10 when 200 *G. m. centralis* were fed on this goat. The goat died on day 15 post-injection. The tsetse flies showed infection rate of 95.8% and were able to infect a clean goat which died on day 18 after the infective feed.

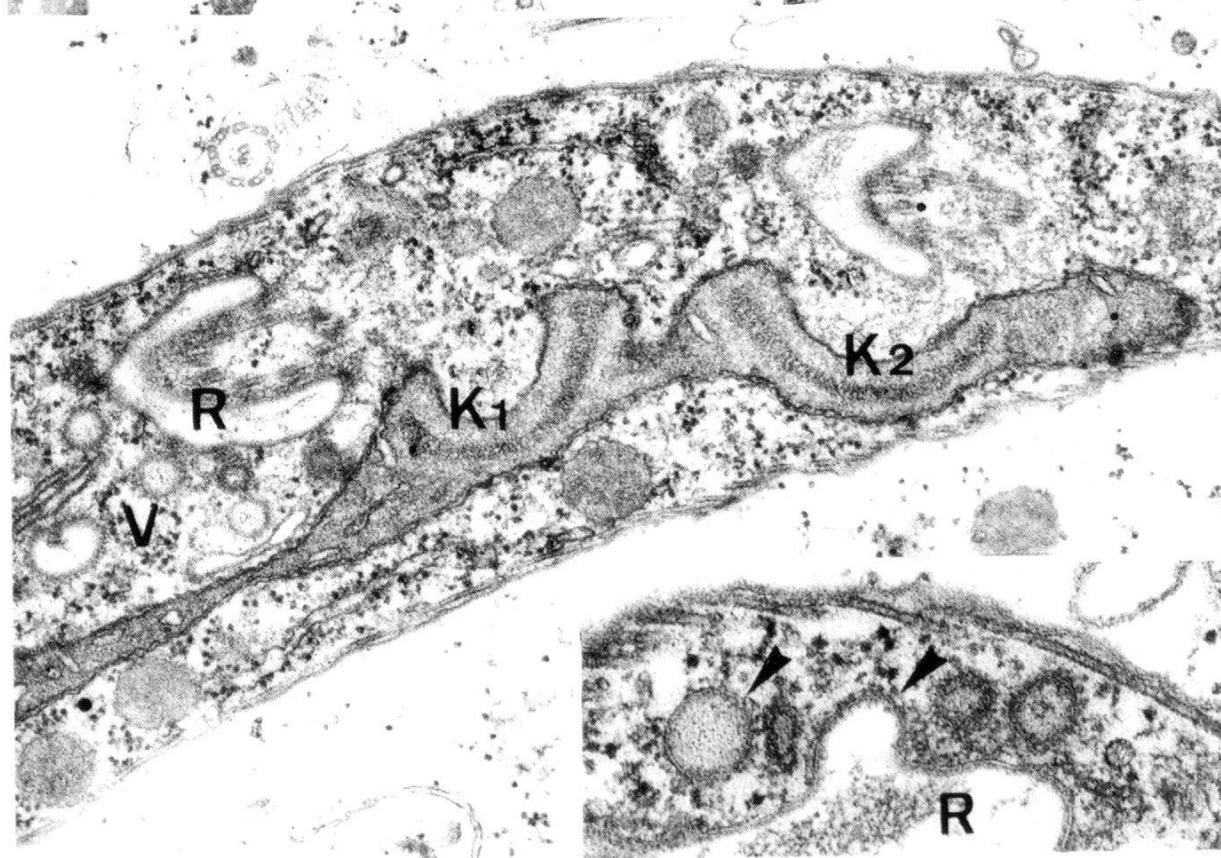
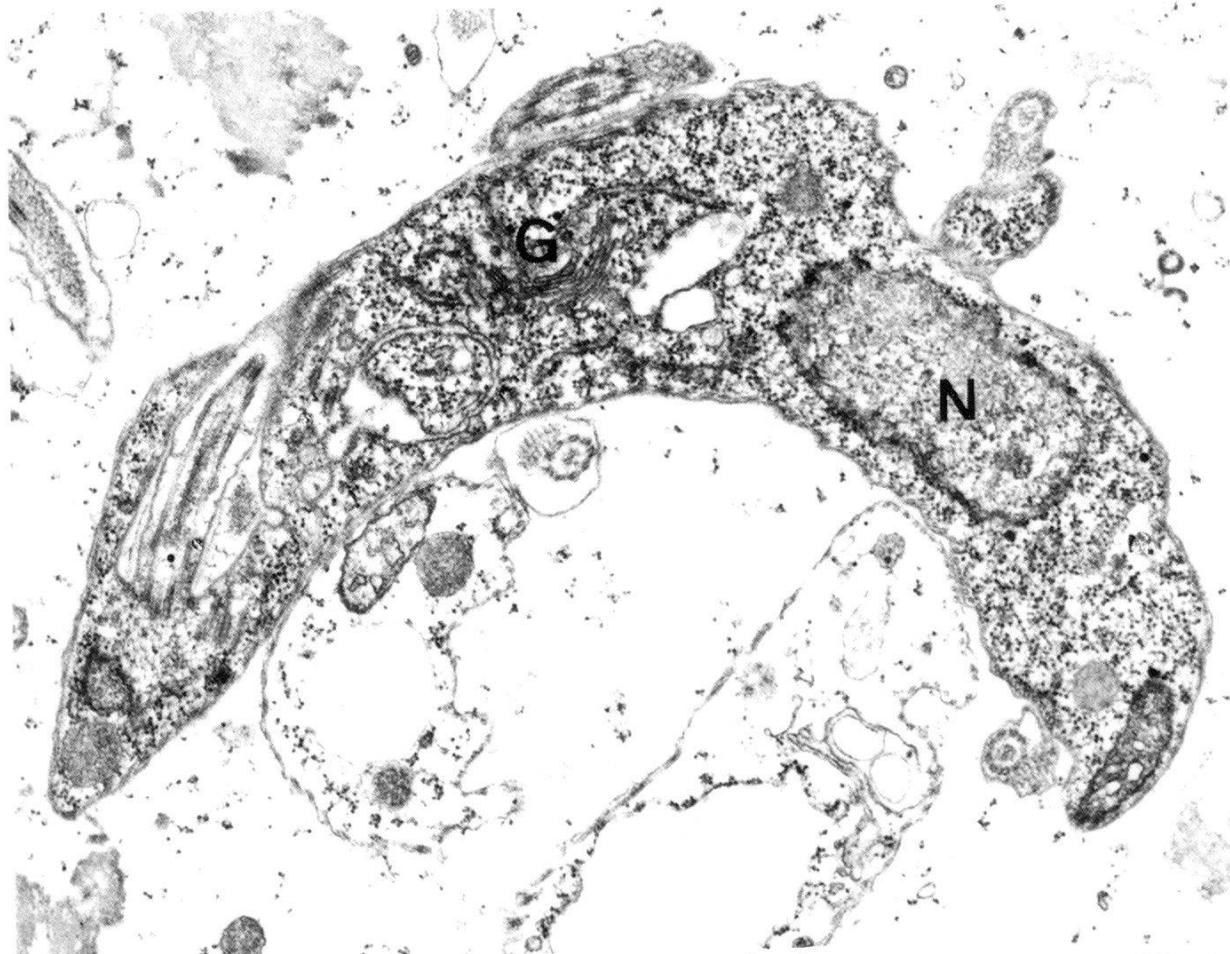
In the electron microscope the typical bowl-shaped *T. vivax* kinetoplast (Vickerman, 1962) could be demonstrated. The mitochondrion was well developed containing many cristae in the mitochondrial matrix. The composition of the cytoplasm reflected a physiologically active cell with large Golgi complex and many membranes belonging to the smooth endoplasmic reticulum. Pinocytotic vesicles could be found near the reservoir. The cells were covered by a light surface coat comparable to the one found in bloodstream forms of *T. vivax* from the goat (Figs. 1-3).

Discussion

The establishment of bloodstream forms of *T. vivax* in culture represents a significant advance in attempts to cultivate this pathogenic trypanosome species in vitro. For 3 months the parasites could be subcultured, were able to infect mammalian hosts, retained their morphological characteristics and virulence, and could very readily be established in *Glossina* from goats infected with the culture forms.

There are only a few reports of cultivation experiments with *T. vivax*.

Fig. 1-3: *Trypanosoma vivax* culture bloodstream forms grown in vitro. 1. Longitudinal section. N. nucleus; G, Golgi complex. 18,000 \times . 2. Dividing cell with the typical bowl-shaped kinetoplast (K₁, K₂) after division. In the vicinity of the reservoir (R) pinocytotic activity and vesicles (V) can be observed. 34,000 \times . 3. Reservoir (R) with pinocytotic vesicles (◀). The unit membrane is covered by a light surface coat. 52,000 \times .



Trager (1959) succeeded in growing procyclic forms in the presence of *Glossina palpalis* organs in a complex medium. The cultures maintained at 30–32°C were not infective to goats. However, infective forms were produced during a 19-h incubation period at the elevated temperature of 38°C. These experiments were repeated, and this time epimastigote forms were observed (Trager, 1975). Isoun and Isoun (1974) attempted to cultivate *T. vivax* in vitro but they managed to maintain the culture for only a few days with increase of trypanosome numbers during the first 2 days. Recently good progress was made on the cultivation of the bloodstream stages of *T. brucei*, *T. rhodesiense* and *T. gambiense* (Brun et al., 1981). Based on these culture systems we succeeded in developing an analogous system that can support the growth of *T. vivax* bloodstream forms.

It should be noted that the stock of *T. vivax* used is infective not only to ruminants but also to mice, rats and rabbits (Moloo, 1981a). This provides an advantage in that infectivity tests of the cultivated *T. vivax* could be carried out with relatively little expense by using small laboratory animals. The present technique should also be tried to cultivate other stocks of *T. vivax* in vitro, and systems need to be developed to allow long-term cultivation of the stages of *T. vivax* which develop in *Glossina*.

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