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In vitro cultivation of *Trypanosoma congolense:* establishment of infective mammalian forms in continuous culture after isolation from the blood of infected mice

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Summary

Bloodstream form trypomastigotes of four cloned stocks of *Trypanosoma congolense* from West Africa were successfully adapted to continuous in vitro culture at 28° C using bovine aorta endothelial cell monolayers and Eagle's minimum essential medium supplemented with 20% normal bovine serum or foetal calf serum. The trypanosomes maintained in vitro morphologically resembled bloodstream forms and remained infective for vertebrate hosts. They also induced local skin reactions in rabbits and were therefore designated "mammalian forms", possibly resembling parasites which develop extravascularly in the vertebrate host following introduction of metacyclic trypanosomes into the skin by bites of tsetse flies. Mammalian forms of two stocks were allowed to transform to procyclic trypanosomes in order to obtain cultures producing epimastigote and metacyclic stages of *T. congolense.* Metacyclic trypanosomes produced in this manner were shown to be neutralized by antiserum raised in rabbits against the homologous trypanosome stock transmitted by tsetse flies.

Key words: *Trypanosoma congolense;* in vitro cultivation; bovine aorta endothelial cell monolayers.

Introduction

In vitro culture systems are a source of specific life cycle stages of trypanosomes, producing numbers of parasites sufficiently large for biochemical and

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immunological analysis. This paper describes the direct adaptation of bloodstream forms of four cloned West African stocks of *Trypanosoma congolense* to continuous culture in the presence of bovine aorta endothelial cell monolayers. Infective trypanosomes of other species can also be maintained in the presence of mammalian cells in cultures derived from parasites isolated from the blood of infected mammalian hosts. Bloodstream forms of *T. brucei*, *T. b. gambiense* and *T. b. rhodesiense* (Brun et al., 1981), *T. vivax* (Brun and Moloo, 1982) and *T. b. evansi* (Zweygarth et al., 1983) have all been grown in vitro in this way.

The entire life cycle of *T. congolense* can be carried out in vitro, in cultures derived from trypanosomes in infected tsetse mouthparts or proventriculi. In the absence of a supporting cell monolayer, they establish as epimastigotes which produce infective metacyclic trypanosomes (Gray et al., 1984). Metacyclic forms induced to transform and divide on mammalian feeder layers can thereafter be grown continuously as infective mammalian forms (Gray et al., 1985).

By changing the culture conditions, *T. congolense* mammalian forms isolated by the methods described in this paper can also be induced to transform to procyclic trypanosomes, and thereafter differentiate to epimastigote and infective metacyclic forms. The life cycle of *T. congolense* therefore, can now also be derived in vitro from bloodstream form populations of this parasite.

Materials and Methods

Trypanosome stocks. Four cloned stocks of *Trypanosoma congolense* from West Africa were used to prepare culture lines. *T. congolense* TREU 1457 and TREU 1467 were derived from Nigerian isolates Zaria/67/LUMP/69 and Ibadan/71/TREU 1095, respectively. TREU 1627 and TREU 1662 were derived respectively from the Gambian isolates Kantong Kunda/77/LUMP/1794 and Koudougo/78/LUMP/1793. Experimental animals were infected with bloodstream forms which had no previous history of in vitro culture.

Hosts. The animals used for production of initial bloodstream forms of *T. congolense* and for infectivity and neutralization tests on cultured trypanosomes were CF1 female mice. Female New Zealand White rabbits, approximately 3 months old, were used to investigate induction of local skin reactions.

Culture conditions. All primary culturing was carried out in Eagle's minimum essential medium (MEM) with Earle's salts and 25 mM HEPES supplemented with 20% (v/v) normal bovine serum (NBS) without antibiotics (MEM-NBS). Other routine culture conditions, the preparation of bovine dermal explants and the isolation and maintenance of bovine aorta endothelial cell feeder layers have been described elsewhere (Gray et al., 1981, 1984, 1985). During the derivation of cultures producing insect stages grown in MEM supplemented with foetal bovine serum (MEM-FBS), passage of trypanosomes to 25 cm^2 "Primaria" flasks obtained from Falcon Tissue Culture Plastics facilitated the adherence of epimastigote forms. The process was also accelerated by the addition of conditioned medium from cultures already producing epimastigotes and metacyclics. Conditioned medium was passaged through 0.22 μm filters before use and added to an equal volume of fresh MEM-FBS.

Preparation of bloodstream forms of T. congolense. Bloodstream trypanosomes collected from mice with parasitaemias of $>10^8$ parasites per ml less than 3 days after infection were found to be the best starting material. Heart blood was collected aseptically into heparin solution (final concen-

tration 5 units per ml). Trypanosomes were separated from other blood components by anion exchange chromatography (Lanham and Godfrey, 1970) and the concentration of parasites in suspension adjusted to 10^7 per ml with MEM-NBS.

Initiation of cultures

a) With a dermal explant. A T-25 culture flask containing a small (5 mm × 5 mm) dermal explant was maintained for several days at 28° C in MEM-NBS. Culture medium was removed and 1 ml of suspension containing 10^7 trypanosomes was added directly to the explant, followed by an equal volume of fresh MEM-NBS. After 2–4 h, the volume of the medium was increased to 4 ml and cultures incubated at 28° C.

b) With a mammalian cell feeder layer. Culture medium was removed from a T-25 flask containing a confluent monolayer of bovine aorta endothelial (BAE) cells and 1 ml of suspension containing 10^7 trypanosomes was added. Fresh MEM-NBS was added gradually over 2–4 h to 4 ml final volume and cultures were incubated at 28° C.

Passage of cultures

Supernatants from primary cultures containing infective *T. congolense* mammalian forms could be introduced on to fresh BAE monolayers in the manner described by Gray et al. (1985). When passaged from established cultures, normally 4×10^6 trypanosomes (1×10^6 per ml) were used to seed fresh monolayers.

Assessment of infectivity

Cultures were examined for growth and infectivity as described previously (Gray et al., 1981). Rabbits were inoculated intradermally with 0.1 ml of parasites in suspension at 10^6 per ml to test the ability of trypanosomes to induce local skin reactions (Luckins et al., 1981). Neutralization tests were carried out by incubating 0.9 ml of a suspension of cultured trypanosomes, containing up to 5×10^5 metacyclic forms of TREU 1457, with 0.1 ml of a $^1/_{10}$ dilution of either normal rabbit serum or serum from a rabbit taken 21 days after infection by a TREU 1457-infected tsetse fly. After 30 min incubation at 4° C, 0.1 ml amounts of suspension were inoculated into groups of 5 mice.

Results

Initiation of mammalian form cultures

1. Cultures established from bloodstream trypanosomes using bovine dermal explant

a) TREU 1457. When transferred to MEM-NBS at 28° C, bloodstream forms of *T. congolense* TREU 1457 aggregated in bundles and adhered to the surface of the culture flask. Most died within the first week in culture and were removed when the medium was changed every 2 to 4 days. After 14 days, by which time fibroblasts had started to grow out from the periphery of the dermal explant, individual trypanosomes could be seen attached by their flagella to the surface of the culture flask. The trypanosomes divided rapidly, attached to the surface of fibroblast cells in the locality of the explant and were morphologically similar to *T. congolense* bloodstream forms. After one month in culture, the numbers had increased so that 5×10^5 mammalian forms could be harvested per ml of culture supernatant every 48 h.

For routine culture, trypanosomes from harvested supernatants were transferred to monolayers of BAE cells in 4 ml MEM-NBS where they attached to the mammalian cell surfaces. Up to 3×10^6 parasites per ml could be harvested every 48 h in culture supernatants, subpassaged on to fresh monolayers and maintained for periods up to six months at 28° C in either MEM-NBS or MEM-FBS.

In MEM-FBS at 28° C, cultured mammalian forms of TREU 1457 on BAE cells could transform into procyclic trypanosomes. Consequently, such cultures were more difficult to maintain than those in MEM-NBS at 28° C, and were therefore not often used as sources of mammalian forms.

b) TREU 1467. When 10^7 TREU 1467 *T. congolense* bloodstream forms were placed beside a dermal explant in 4 ml MEM-NBS at 28° C, trypanosomes quickly established themselves in vitro in exactly the same way as those of TREU 1457. After 29 days in vitro, 2×10^5 organisms were transferred to a BAE cell monolayers and, after a further 21 days, 10^6 trypanosomes per ml could be harvested from culture supernatants. On changing the culture medium to MEM-FBS the number of mammalian forms in the culture increased dramatically and as many as 1.5×10^7 trypanosomes per ml were harvested at each medium change. Unlike TREU 1457, trypanosomes of this stock did not readily transform into procyclic forms.

c) TREU 1662. This stock was adapted to continuous culture several times and, on one occasion, only 3×10^5 bloodstream forms were used as initial starting material. After 31 days in vitro sufficient trypanosomes had established for 6×10^5 mammalian forms to be transferred to a BAE cell monolayer for routine maintenance.

TREU 1662 was also maintained at 28° C and at 37° C in MEM-FBS. There were fewer procyclic forms than in cultures of TREU 1457.

2. Cultures established from bloodstream trypansomes using a bovine aorta endothelial cell monolayer

Bloodstream forms of TREU 1457, TREU 1467 and TREU 1662 were all introduced directly onto a BAE cell monolayer at the same time as they were placed beside the dermal skin explant. Gradually all trypanosomes died and continuous mammalian form cultures of these stocks were not produced by this method. For stock TREU 1627, however, direct placement of bloodstream trypanosomes onto a BAE monolayer was more successful in initiating cultures of trypanosomes than the use of a dermal explant.

Continuous cultures of TREU 1627 mammalian forms were obtained by placing bloodstream trypanosomes directly on to BAE cells. The trypanosomes became very lightly attached to the surface of the cells and only a few aggregated in the supernatant. Very active trypanosomes closely associated with, but not attached to, the feeder cells were lost during medium changes. Consequently, the time taken for the number of trypanosomes to increase sufficiently to estab-

Table 1. Infectivity of mammalian forms of *T. congolense* maintained in vitro on BAE cells

Stock	Culture conditions	Months in culture at time of infectivity test	Log number of trypanosomes per ml	Log number of ID_{63} per ml and SE
TREU 1457	28° C, MEM-NBS	3	6.1	3.8 ± 0.3
	28° C, MEM-NBS + dermal explant	3	5.6	4.1 ± 0.5
	28° C, MEM-FBS	3	5.9	4.8 ± 0.4
TREU 1662	28° C, MEM-NBS	3	5.6	4.1 ± 0.5
	28° C, MEM-NBS	5	5.4	3.8 ± 0.5
	37° C, MEM-NBS	5	5.5	4.6 ± 0.3
TREU 1627	28° C, MEM-NBS	3	6.0	5.1 ± 0.3

lish the culture was 12 weeks, when the number of trypanosomes in the supernatant reached $>1 \times 10^5$ per ml and the culture could be routinely passaged. Mammalian forms of TREU 1627 are shown in Fig. 1a.

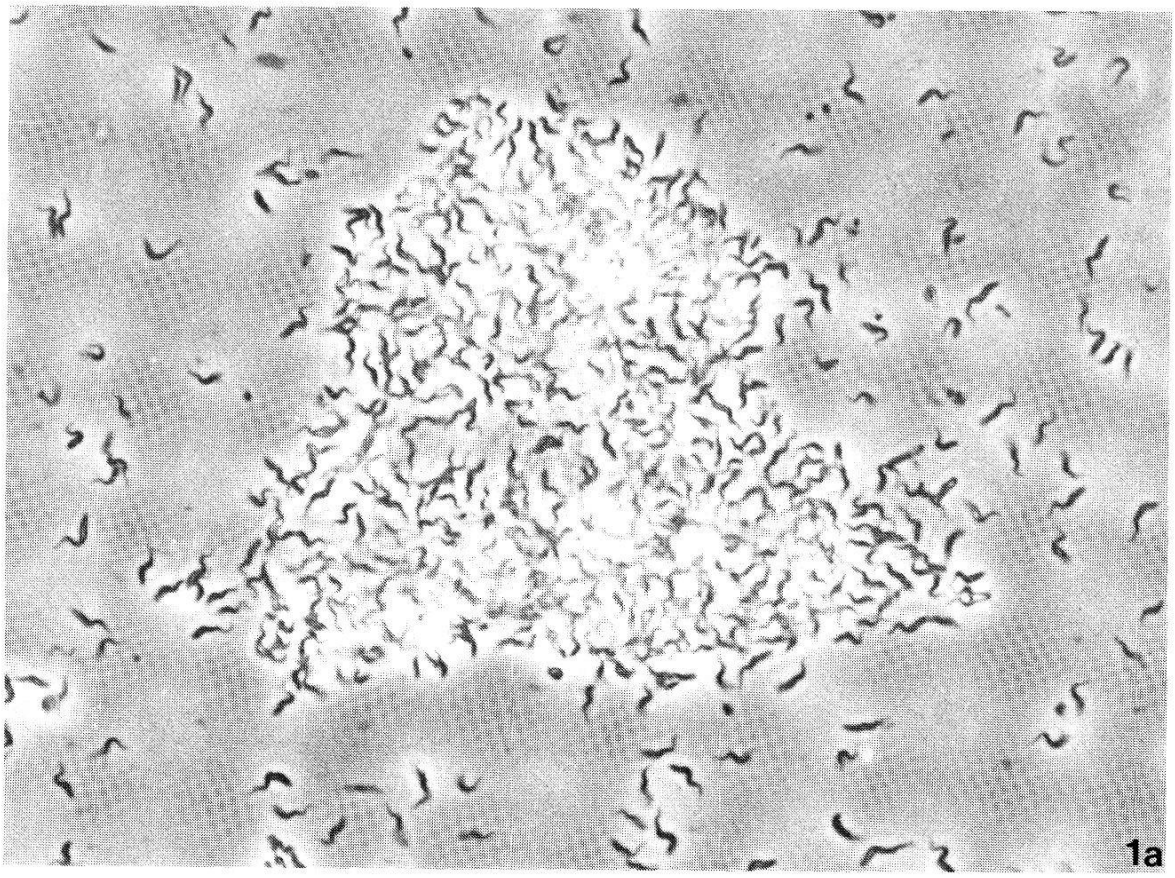
In MEM-FCS at 28° C, TREU 1627 mammalian forms grew to high density on the feeder cells and 1.5×10^7 per ml could be harvested at each medium change. Like TREU 1457, TREU 1627 mammalian forms transformed into procyclic trypanosomes especially if maximum density was reached before medium change.

Several attempts to culture TREU 1627 for long periods in MEM-FBS or MEM-NBS at 37° C were unsuccessful.

Cultures of mammalian forms of all stocks maintained indefinitely *in vitro* retained their infectivity for mice. Examples of the range obtained by infectivity titrations are shown in Table 1, calculated by the method of Lumsden et al. (1963). All stocks were successfully cryopreserved according to the method of Gray et al. (1984, 1985).

Derivation of cultures producing infective metacyclic forms

The ability of mammalian forms of TREU 1457 and TREU 1627 to transform to procyclic forms when transferred to MEM-FBS was exploited when attempts were made to culture insect forms of these two stocks. By scraping away part of the BAE monolayer, newly differentiated epimastigote trypanosomes could be made to adhere to the surface of the culture flask. Cultures were maintained with 48 h changes of MEM-FCS until epimastigotes were established as thick bundles on the floor of the culture flask. During this period, cultures which consisted of procyclic and epimastigote forms were not infective to mice. Infectivity was regained with the appearance of mobile metacyclic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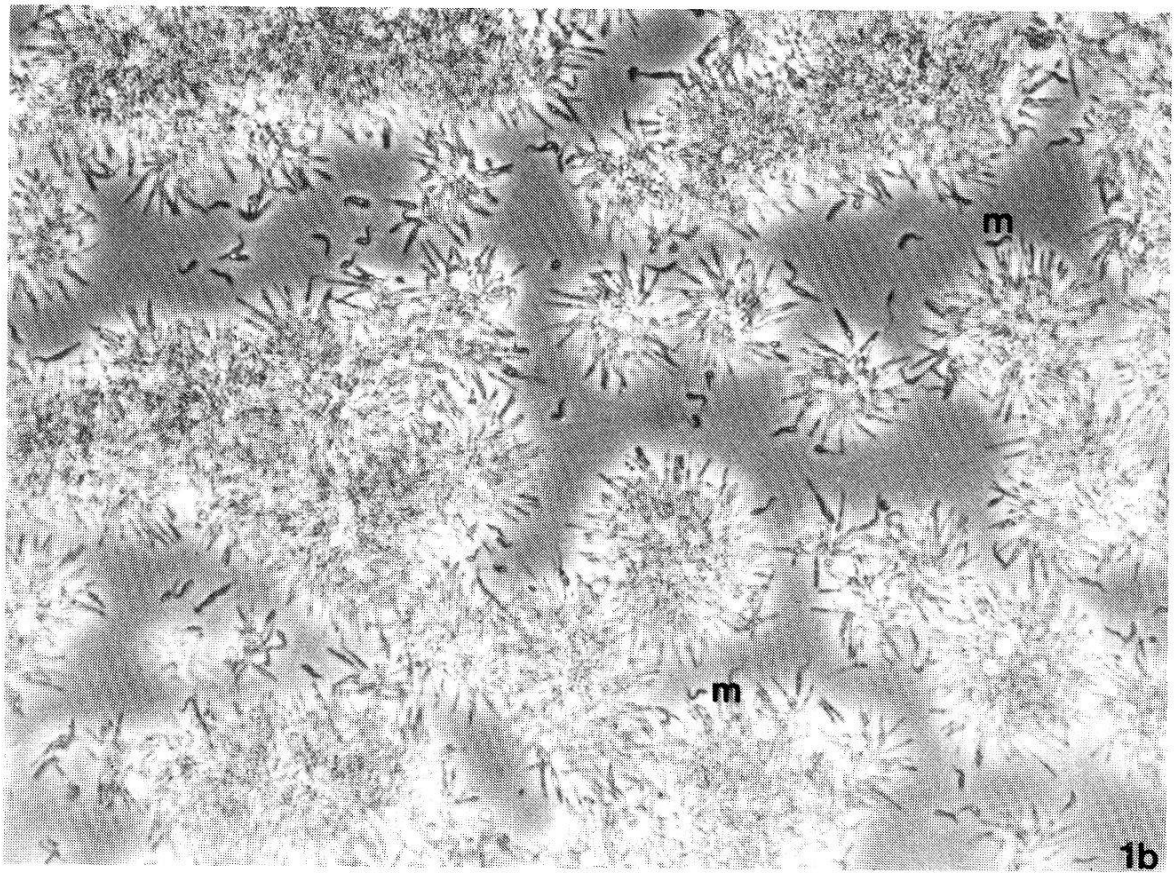
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Table 2. Neutralization of in vitro derived TREU 1457 metacyclics

T. congolense stock	Source of infected material for primary isolation	Inoculum metacyclics $\times 10^{-3}$	Mice infected after incubation of metacyclics with antiserum	
			PI	21d
TREU 1457	tsetse tissue	10	5/5	0/5
		50	5/5	0/5
	mouse blood	10	4/5	0/5
		50	5/5	0/5

PI = preinfection rabbit serum
21d = rabbit serum collected 21 days after fly bite

trypanosomes between bundles of epimastigote forms. This process could take 2–12 weeks.

Cultures of insect stages derived in this way (Fig. 1b) were morphologically identical to those derived from infected tsetse flies (Gray et al., 1984).

Neutralization test

The results of the neutralization test are shown in Table 2. In vitro derived metacyclics were neutralized by rabbit serum collected 21 days after the rabbit had been bitten by a tsetse fly infected with TREU 1457. Different sources of trypanosomes used for initial primary cultures, i.e. tsetse tissues or mouse blood, did not affect the nature of the antigens expressed in resulting metacyclic populations. Preinfection rabbit serum, however, failed to protect and all but one mouse in the control groups became infected.

Induction of local skin reactions

In vitro derived forms of the four stocks of *T. congolense* isolated from infected mouse blood were tested for their ability to induce local reactions when injected intradermally into the skin of susceptible normal rabbits. Cultured mammalian forms of TREU 1457, TREU 1467 and TREU 1627 induced local reactions, but TREU 1662 did not. In vitro derived metacyclics of TREU 1457 and TREU 1627 also induced such reactions. Bloodstream forms of each stock failed to induce local skin reactions.

Fig. 1.
a) Bovine aorta endothelial (BAE) feeder cells with mammalian forms of *T. congolense* TREU 1627 growing on the cells and the surface of the culture flask. This micrograph of an unfixed preparation was taken with an inverted phase contrast microscope. Magnification ×300.
b) Phase contrast micrograph of insect forms of TREU 1627 derived from a culture of mammalian forms. No BAE cells are present. Bundles of epimastigote forms, firmly attached to the floor of the culture flask, are surrounded by infective metacyclic trypanosomes (m). Magnification ×240.

Discussion

In cultures of *T. congolense* mammalian forms, trypanosomes grew lightly attached to the upper surfaces of the feeder layer cells, and could be removed by gentle or persistent pipetting. Similar observations have been made for an uncloned East African isolate of *T. congolense* adapted to culture at 37° C on confluent BAE cells (Hirumi and Hirumi, 1984). In our experiments a confluent monolayer was not essential to ensure a successful trypanosome culture and after inadvertent loss of endothelial cells during medium change residual cells divided to re-cover the flask surface. Removal of all cells, however, drastically inhibited trypanosome division. Fig. 1a shows that, in non-confluent monolayers, the density of trypanosomes growing on BAE cells was much greater than on the surrounding flask surface. Since trypanosome distribution throughout the culture flask was rarely uniform, it was impossible to estimate accurately the growth rate of these forms in culture.

Mammalian forms of all four cloned West African stocks described in this paper were morphologically indistinguishable. There were differences between stocks, however, in the maximum density of parasites obtained under the standard culture conditions described, in the ability of the mammalian forms to transform to procyclic forms, in their ability to grow at 37° C and in the strength of attachment of trypanosomes to their feeder cells.

Trypanosomes detached from the feeder layer in large numbers only in cultures where maximum trypanosome density had been reached before medium was changed. Intercellular pockets of parasites were occasionally found in cultures in which trypanosome density had been consistently high. This behaviour contrasts with that of cultures of infective forms of *T. brucei* subspecies (Brun et al., 1981) and *T. vivax* (Brun and Moloo, 1982), in which the majority of trypanosomes grew in suspension in the culture medium, or in intercellular pockets within the feeder layer. It has been suggested that, with *T. brucei* cultures, the intercellular population is necessary for continuous growth of the whole culture (Tanner, 1980; P. Dukes, personal communication), but no similar property was observed in cultures of *T. congolense.*

Derivation of infective insect-form cultures of *T. congolense* producing metacyclic trypanosomes from infective mammalian forms growing on a cell feeder layer has already been demonstrated (Gray et al., 1985). However, using trypanosomes isolated from infected mouse blood we have been able to grow all stages of the life cycle of this parasite without any requirement for tsetse tissue, which may not be readily available in many laboratories. Metacyclic populations of TREU 1457 derived from either tsetse tissue or from mouse blood were both neutralized by serum from rabbits infected by bites of *G. morsitans* carrying TREU 1457, and we conclude that the organisms produced by these different culture techniques are effectively the same as those developed in tsetse flies.

Metacyclics of TREU 1457 and TREU 1627 produced in culture induced local skin reactions when inoculated into rabbits, which is a property they share with metacyclics from natural infections and with extravascular forms found within reaction sites but not with true bloodstream forms (Luckins and Gray, 1983). Tsetse transmitted metacyclic trypanosomes of TREU 1662 did not produce local reactions (unpublished observations) and it is interesting that neither did cultured mammalian forms of this stock. The cultured mammalian forms of TREU 1457, TREU 1467 and TREU 1627, however, had acquired the ability to cause local reactions, although they were morphologically indistinguishable from the bloodstream trypanosomes from which they were derived. This implies that an intrinsic property of bloodstream forms of *T. congolense* which have adapted to culture is the ability to induce local reactions when injected intradermally into susceptible hosts. Mammalian forms produced when cultured metacyclic trypanosomes of TREU 1457 transform and divide on BAE cells also have this ability (Gray et al., 1985).

There is no information on whether *T. congolense* trypanosomes adapted to continuous culture at 37° C by Hirumi and Hirumi (1984) could induce local skin reactions. These cultures, however, were derived from 25% of the original bloodstream form population, while, in our experiments, only a very tiny proportion of bloodstream trypanosomes adapted to growth in vitro at 28° C. Trypanosomes used in our experiments established much more readily at 28° C than at 37° C. Different subpopulations of *T. congolense* bloodstream forms, therefore, may be growing in these two culture systems. Both methods of isolation, using bloodstream trypanosomes of *T. congolense* derived from a stock known to produce local skin reactions in mammals after bite by infected tsetse, could be carried out. A comparison of the properties of both types of cultured bloodstream forms, including their ability to induce local skin reactions, would then be possible.

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