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In vitro cultivation of *Trypanosoma congolense*: the production of infective forms from metacyclic trypanosomes cultured on bovine endothelial cell monolayers

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Summary

After transfer to bovine endothelial cell monolayers cultured in Eagle's minimal essential medium at 28°C or 37°C metacyclic trypanosomes of three cloned stocks of *Trypanosoma congolense* became morphologically similar to parasites found in the bloodstream of the vertebrate host. The trypanosomes resumed division and grew in close association with the mammalian cells, which were essential for growth. These dividing infective forms had the ability to cause local skin reactions and systemic infections when inoculated intradermally into rabbits. Trypanosomes grown in medium supplemented with foetal calf serum (FCS) eventually differentiated into procyclic forms. No such change occurred in medium supplemented with normal bovine serum. If procyclic forms in FCS were allowed to continue their differentiation at 28°C they eventually produced epimastigotes which gave rise to infective metacyclic trypanosomes once more. It was thus possible to grow and maintain several different developmental stages of *T. congolense* by varying culture conditions.

Key words: *Trypanosoma congolense*; clone; in vitro cultivation; mammalian forms; bovine endothelial cell monolayer.

Introduction

We have shown that several different stocks of *Trypanosoma congolense* can be maintained in vitro for long periods in the absence of a supporting cell

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monolayer (Gray et al., 1984). In such cultures large numbers of epimastigote trypanosomes are produced which give rise to the metacyclic forms retaining the morphological, biological and antigenic characteristics of the metacyclic trypanosomes found in the proboscis of the tsetse fly (Gray et al., 1981, 1984; Luckins et al., 1981; Crowe et al., 1983).

Infective forms of pathogenic trypanosomes may also be cultured in the presence of mammalian cells. Bloodstream forms of *T. brucei* and *T. vivax* have been successfully cultured with feeder layers of mammalian cells from their vertebrate hosts (Hirumi et al., 1977; Hill et al., 1978; Brun et al., 1979, 1981; Brun and Moloo, 1982) and from metacyclic forms of *T. brucei* (Brun et al., 1979, 1981). Attempts, however, to maintain infectivity of *T. congolense* on fibroblast monolayers were previously unsuccessful (El-on et al., 1977) until Gray and Luckins (1982) and Hirumi and Hirumi (1984) introduced culture procedures using endothelial cell monolayers.

During the initial stages of infection with cyclically transmitted *T. congolense* trypanosomes establish extravascularly in the collagen (Luckins and Gray, 1978) and such forms have been used to initiate cultures of *T. congolense* which remained infective for up to three weeks (Gray et al., 1979). More recently, we have shown that in cultures of *T. congolense* established from the mouth-parts of infected *Glossina morsitans* trypanosomes morphologically similar to those found in the mammalian host can multiply in close association with out-growths of fibroblast cells from normal bovine collagen (Gray et al., 1984). Metacyclic trypanosomes may thus be suitable organisms to initiate cultures of mammalian stages of *T. congolense*, as has been demonstrated using *T. brucei* (Brun et al., 1981). The following paper describes the culture of *T. congolense* in association with bovine endothelial monolayers using both *in vivo* and *in vitro* derived metacyclic trypanosomes.

Materials and Methods

Trypanosome stock. Trypanosomes used to initiate cultures were obtained from three cloned stocks of *T. congolense*, TREU 1457, TREU 1468 and TREU 1662. The histories of the clones TREU 1457 and TREU 1468 have been described elsewhere (Luckins and Gray, 1983). TREU 1662 is a cloned stock of LUMP 1783. TREU 1457 from West Africa (WA) and TREU 1468 from East Africa (EA) have been classified on the basis of their isoenzyme characteristics as belonging to a savannah zymodeme and TREU 1662 also from West Africa (WAR) as belonging to a riverain zymodeme (Young and Godfrey, 1983). Cultures were designated with the number of tsetse fly from which they were established, the two stocks TREU 1457 and 1468 being identified by the prefix WA and EA, respectively, and TREU 1662 by the prefix WAR.

Culture conditions. All cultures were maintained at 28°C in Eagle's minimal essential medium (MEM) with Earle's salts and 25 mM HEPES supplemented with 20% heat inactivated foetal calf serum (MEM-FCS) as described by Gray et al. (1981). In addition MEM was also supplemented with 20% normal bovine serum (MEM-NBS) obtained commercially or from Jersey or Friesian cattle kept at this laboratory. Antibiotics were not used for the routine maintenance of cultures.

Establishment of bovine aorta endothelial cell line (BAE). The bovine cell monolayer was initiated by gently scraping with a scalpel blade surface endothelial cells from the aortic intima of a

12-month-old Friesian calf, washing them in phosphate buffered saline pH 7.2 and incubating them at 37°C in 4 ml of MEM-FCS in 25 cm² culture flasks gassed with 5% CO₂ in air. Flasks in which clonal development of endothelial-like cells was evident and in which no fibroblasts were seen were selected, and established islands of cells were removed with a silicon rubber "policeman", after one week and placed in a fresh flask; and after four such weekly serial passages, a uniform monolayer of endothelial-like cells was obtained which could then be sub-cultured and passaged using versene/ trypsin. The cells were cryopreserved at the 7th passage using 10% DMSO after 8 weeks in culture. This cell line had a finite life and was used up to passage 26. Endothelial cell cultures remained stable at pH 7.3 for one week without change of medium.

Establishment of trypanosome cultures

1. *Metacyclic trypanosomes from infected tsetse flies.* Tsetse flies were infected and maintained as described previously (Gray et al., 1981). Trypanosomes were obtained from the detached proboscis or the saliva of infected flies.

a) *Proboscis.* The proboscis of the fly was removed from the head and placed beside either a bovine dermal collagen explant (Gray et al., 1981) or on a BAE monolayer in 4 ml MEM-FCS or MEM-NBS.

b) *Saliva.* Infected tsetse flies were induced to probe into 50 µl of medium as described by Brun et al. (1979). After confirming that metacyclic trypanosomes were present, the pool was placed beside a dermal collagen explant from which medium had been removed after 48 h incubation at 28°C or on a BAE monolayer used 4–6 days after passage. It was then left for 30 min before 4 ml of fresh MEM-FCS was added.

2. *Metacyclic trypanosomes from cultures.* The infective, metacyclic trypanosomes produced in continuous cultures of insect forms (Gray et al., 1981, 1984) were separated on DE-52 anion exchange columns. BAE monolayers were seeded with 1 × 10⁵–8 × 10⁶ of these forms, and fresh MEM-FCS or MEM-NBS was added to make the final volume 4 ml.

Passage of cultures

1. *From dermal collagen explants.* Supernatants from primary cultures containing up to 4 × 10⁶ trypanosomes were removed and placed on either a 4 to 6-day-old BAE monolayer or a dermal collagen explant. Fresh medium was added to 4 ml final volume.

2. *From BAE monolayers.* Supernatants were removed from primary cultures and trypanosomes washed off monolayers with fresh medium. New BAE monolayers were seeded with up to 4 × 10⁶ trypanosomes. Complete medium changes were made at 48 h intervals, but sufficient parasites were left so that near maximum trypanosomes density was attained by the time of the following medium change.

Assessment of growth characteristics and induction of local skin reaction

Cultures were examined for growth and infectivity as described previously using thin films of supernatants, fixed, hydrolysed and stained with Giemsa (Gray et al., 1984). Rabbits were inoculated intradermally with culture supernatants to test the ability of culture trypanosomes to induce local skin reactions (Luckins et al., 1981).

As a control, *Glossina morsitans* infected with *T. congolense* TREU 1457 were allowed to feed on the backs of two New Zealand White rabbits and on day 7, when local skin reactions had developed, the skin at the bite site was pricked and a small amount of exudate was diluted with 0.2 ml of phosphate buffered saline, pH 7.2, and 0.1 ml amounts inoculated intradermally into the skin of two more New Zealand White rabbits.

Electron microscopy (EM)

For transmission electron microscopy endothelial cells and trypanosomes were scraped from culture flasks, concentrated by centrifugation and then fixed for 1 h in 2.5% glutaraldehyde in 0.1 M

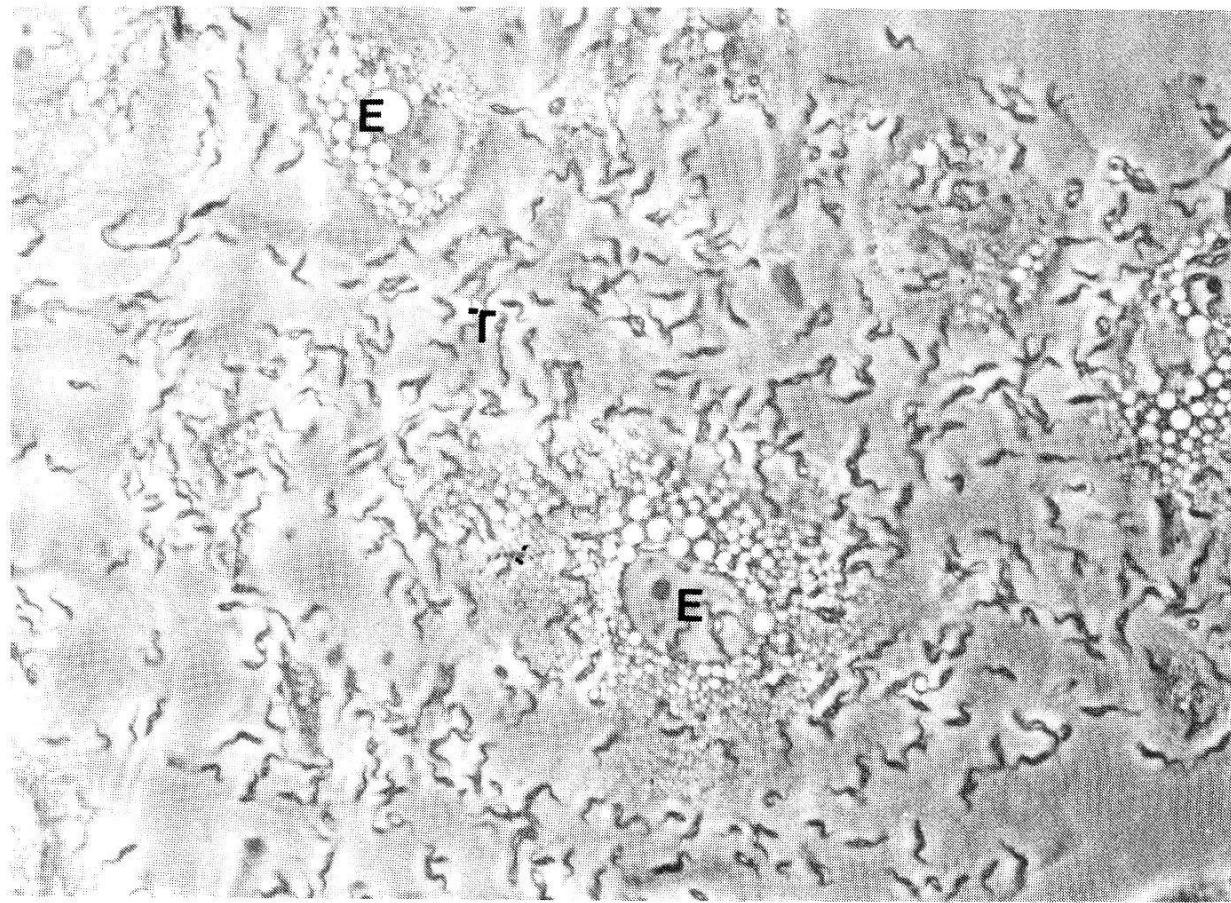


Fig. 1. *Trypanosoma congolense* TREU 1457 growing on bovine aorta endothelial cell monolayers ($\times 300$). E = endothelial cell monolayer; T = trypanosome.

phosphate buffer, pH 7.4, containing 20 mg/ml CaCl_2 . They were then processed for electron microscopy using techniques described by Tetley et al. (1981). For scanning electron microscopy BAE-monolayer cells were grown on Thermanox coverslips (Lux Scientific Corp.) in culture flasks seeded with trypanosomes and washed gently with MEM and then fixed with glutaraldehyde. Thereafter the material was processed according to procedures described by Vickerman and Tetley (1977).

Results

Dividing trypanosomes of three stocks of *T. congolense* were continuously propagated in vitro in the presence of bovine aorta endothelial cells at 28 °C (Fig. 1). Cultures could be initiated either with metacyclic trypanosomes from tsetse flies or with metacyclines produced in culture. Trypanosomes grew in close association with the cells on which they were maintained; the cells appeared essential for their growth. The parasites attached to the cells by their flagellar end but this attachment was not strong and they could be washed from the cells by expelling medium from a pipette over the monolayer. These forms of *T. congolense* were infective for laboratory animals and were indistinguishable morphologically from those found in the bloodstream of the mammalian host.

Table 1. Infectivity of different stocks of *Trypanosoma congolense* maintained on bovine aorta endothelial cells (BAE)

Trypanosome stock	Culture and passage number	Medium supplement*	Maintenance temperature (°C)	Passage number of BAE monolayer	Age of culture (days)	Total time of stock in vitro (days)	Log ₁₀ total number of trypanosomes tested	Log ₁₀ number of infective organisms (ID ₆₃ s/ml)
TREU 1662	WAR69/BAE	FCS	28	—	25	25	5.5	4.8 ± 0.3
	WAR69p1/BAE	FCS	28	14	13	45	5.9	4.8 ± 0.3
	WAR69p2/BAE	FCS	28	16	23	45	5.9	5.0 ± 0.3
	WAR69p2/BAE	FCS	28	16	42	94	6.1	4.8 ± 0.3
	WAR69p2/BAE	FCS	28	16	123	173	5.9	2.6 ± 0.3
	WAR69p3/BAE	FCS	28	18	125	173	6.1	4.4 ± 0.5
	WAR69p7/BAE	FCS	28	9	30	173	5.9	4.8 ± 0.3
	WAR69p2/BAE	FCS	37	17	31	76	5.9	4.0 ± 0.3
	WAR69p2/BAE	FCS	37	17	51	96	6.0	3.3 ± 0.3
	WAR69p2/BAE	FCS	37	17	128	173	5.9	3.1 ± 0.3
	WAR69p3/BAE	FCS	28	18	14	73	6.1	4.3 ± 0.3
	WAR69p5/BAE	FCS	28	20	22	65	6.0	3.7 ± 0.3
	WAR69p10/BAE	FCS	28	12	13	123	5.9	3.8 ± 0.3
	WAR69p15/BAE	FCS	28	12	12	173	6.1	2.9 ± 0.3
	WAR69p2/BAE	NBS	28	15	57	105	6.1	4.1 ± 0.3
	WAR69p2/BAE	NBS	28	15	125	173	6.0	3.6 ± 0.3
	WAR69p4/BAE	NBS	28	9	30	173	5.8	3.9 ± 0.3
	WAR69p3/BAE	NBS	37	19	24	102	5.5	3.6 ± 0.3
	WAR69p7/BAE	NBS	37	12	12	173	6.0	3.6 ± 0.3
TREU 1468	EA44/BAE	FCS	28	20	7	7	5.9	4.0 ± 0.3
	EA44/BAE	FCS	28	20	45	45	6.1	3.0 ± 0.5
	EA44/BAE	NBS	28	20	71	71	6.7	3.6 ± 0.5
	EA1/BAE	NBS	28	20	35	35	5.5	4.1 ± 0.5
TREU 1457	WA22p1/BAE	FCS	28	22	10	14	5.9	2.8 ± 0.5
	WA22p1/BAE	FCS	28	9	21	55	5.8	5.0 ± 0.3
	WA22p2/BAE	NBS	28	22	9	9	5.8	2.8 ± 0.5
	WA22p1/BAE	NBS	28	26	33	50	6.4	3.4 ± 0.3
	WA22p4/BAE	NBS	28	14	16	105	5.4	3.0 ± 0.3

* FCS = foetal calf serum; NBS = normal bovine serum (both at 20%).

1. Cultures established with metacyclic trypanosomes

a) *From proboscis of tsetse fly infected with TREU 1662.* Trypanosomes resembling bloodstream forms were seen in close proximity to a skin explant in MEM-FCS, 14 days after placing beside in the proboscis of tsetse fly WAR69 infected with TREU 1662. These trypanosomes, which we designate "mammalian forms", increased in numbers, adhering to the bottom of the culture flask or to fibroblast outgrowths from the dermal collagen explant. On day 25 the culture was found to be infective for mice (Table 1). This culture continued to produce infective trypanosomes but these were gradually outgrown by procyclics which appeared after 45 days. By the end of the observation period (day 161), mammalian forms accounted for less than 1% of the total in culture.

Thirty-two days after initiation, 3×10^6 trypanosomes from WAR69 were also seeded onto a BAE monolayer in MEM-FCS at 28°C (WAR69p1/BAE). Dividing mammalian form trypanosomes were seen in close association with the cells but forms with the morphological characteristics of procyclics developed in the supernatant. These procyclics were reduced in number at each medium change by washing with an extra 2 ml medium before the final 4 ml was added. On longer cultivation, however, procyclics began to develop in endothelial layer intercellular spaces and had overgrown the culture by day 58 when it was discarded. During this period the culture was passaged at 13, 15 and 21 days to start, respectively a line in MEM-FCS at 37°C , another line in MEM-NBS at 28°C and to continue the line in MEM-FCS at 28°C .

In the line maintained at 37°C , it was necessary to passage the trypanosomes at weekly intervals to prevent the development of intercellular pockets of procyclics. At its second passage a culture was initiated at 37°C using MEM-NBS. The line in MEM-NBS never developed procyclic forms although it was cultured for 128 days before being passaged.

Trypanosomes maintained in MEM-FCS and MEM-NBS at 28°C were serially passaged monthly for 5 and 6 months, respectively. Procyclics were never seen in MEM-NBS, but they did develop in culture in MEM-FCS, in the endothelial cell layer intercellular spaces, although they never outgrew the infective forms.

Several culture lines of WAR69 were continuously maintained using MEM supplemented with FCS or NBS at both 28° and 37°C (Table 1).

b) *From saliva from a tsetse fly infected with TREU 1468.* Metacyclic trypanosomes extruded by tsetse fly EA1 infected with TREU 1468 were cultured at 28°C in MEM-NBS containing a dermal collagen explant. A few trypanosomes were observed in the vicinity of the explant for the first 14 days but then none were seen for 10 days. On day 14 trypanosomes were seen once more and increased in number until by day 30, $8 \times 10^4/\text{ml}$ were present. At this time they were adhering to and dividing on fibroblastic cells emanating from the skin explant. From day 34, the numbers increased to $3 \times 10^5/\text{ml}$ and then remained at this level until the end of the observation period on day 66.

Table 2. Establishment of cultures of mammalian form *Trypanosoma congolense* TREU 1468 on bovine aorta endothelial BAE monolayers using cultured metacyclic trypanosomes

Numbers of metacyclic trypanosomes	Medium [△]	Passage number of BAE monolayer	Number of trypanosomes harvested from culture supernatants	Observation period (days)
EA44p1*, 1 × 10 ⁶	MEM/FCS	14	Procyclic forms only	37
EA44p7*, 3 × 10 ⁵	MEM/FCS	14	3 × 10 ⁶ /ml	117
	MEM/NBS			
EA44p9*, 2 × 10 ⁶	MEM/FCS**	20	2.5 × 10 ⁶ /ml	70
	MEM/NBS			
EA31p5*, 1 × 10 ⁵	MEM/FCS**	20	2 × 10 ⁶ /ml	57
	MEM/NBS			

* Designation and passage number of cultured metacyclic trypanosomes.

** Culture medium was initially MEM/FCS but this was later replaced with MEM/NBS in order to control development of procyclic trypanosomes.

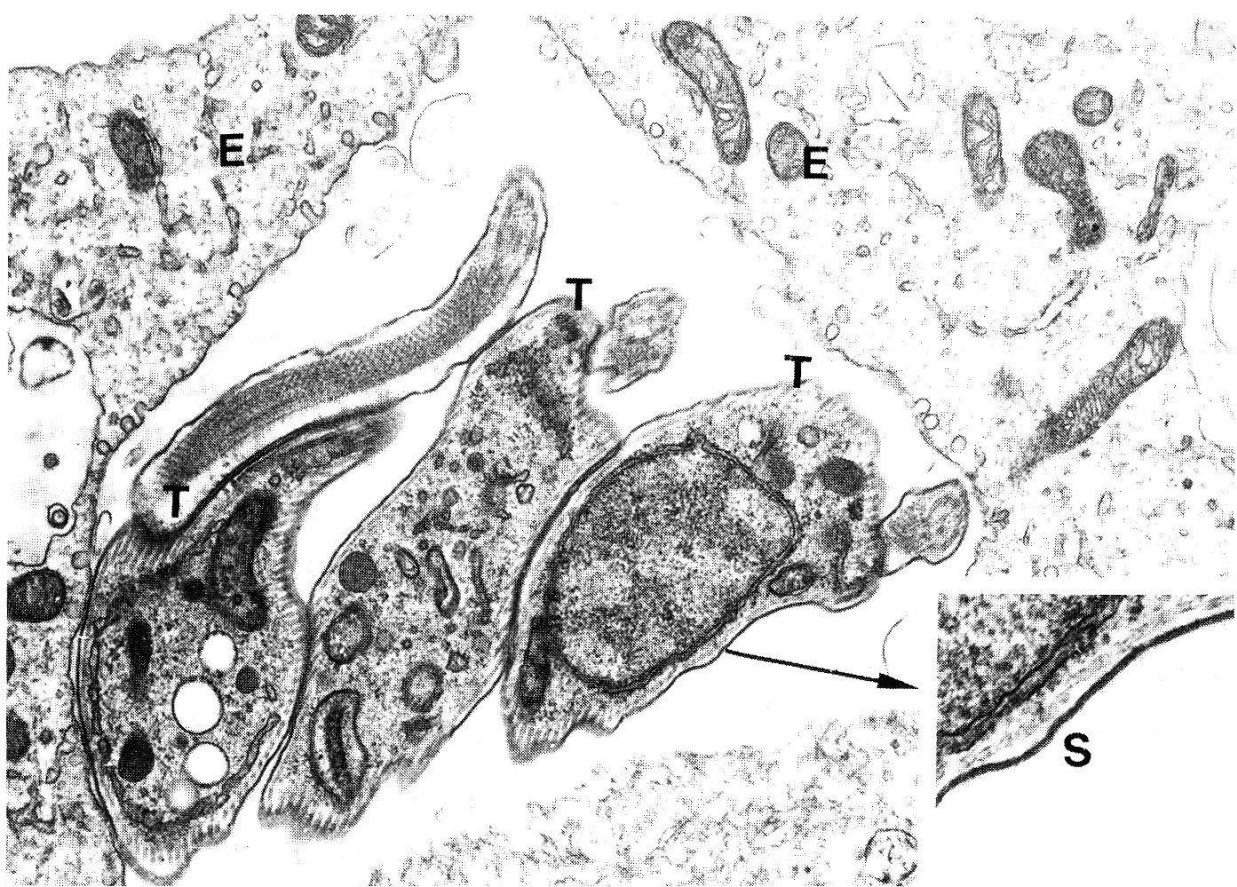
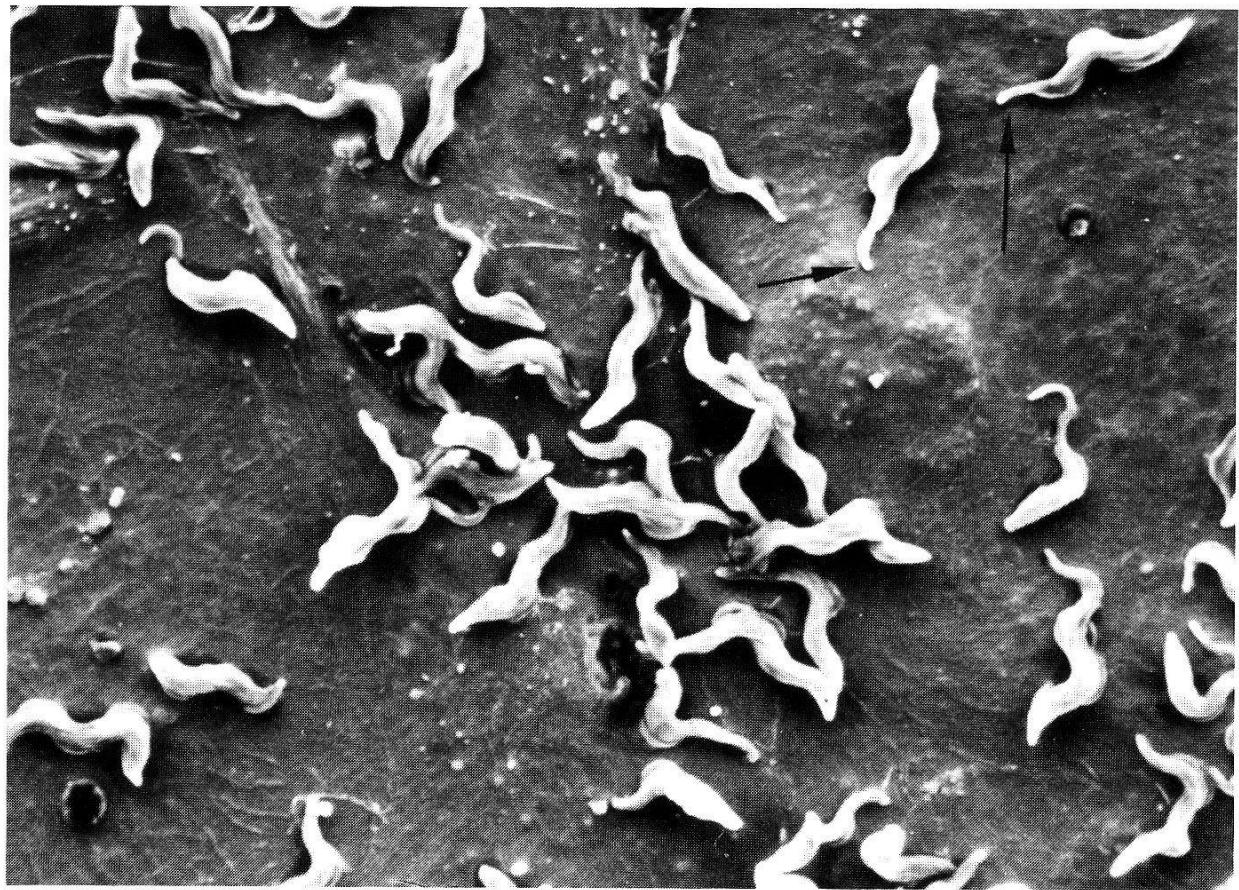
△ FCS = foetal calf serum; NBS = normal bovine serum.

Trypanosomes from the primary culture EA1 were passaged onto BAE monolayers in MEM-NBS on several occasions between day 21 and day 54. None of these passages were maintained beyond 18 days due to gradual decline in trypanosome numbers.

Three attempts were made to culture TREU 1468 metacyclines from saliva directly onto BAE monolayers. Starting from 10 and 50 metacyclines, respectively, trypanosomes increased in number up to day 14 in vitro but thereafter the numbers declined and at day 21 no trypanosomes could be seen. Four metacyclines were used in another attempt but these differentiated into procyclics and the culture was not infective for mice during its observation period of 63 days.

2. Cultures established from metacyclic trypanosomes grown in vitro

a) *TREU 1468*. Four attempts were made to initiate cultures of infective trypanosomes at 28°C using metacyclic forms of TREU 1468 produced in vitro. These procedures are summarized in Table 2. Metacyclic trypanosomes rapidly adhered to the endothelial cell monolayer and commenced dividing. In the presence of MEM-FCS however, procyclic forms began to appear and in one instance became the predominant form in culture by day 37. In the remaining cultures the medium supplement was changed to MEM-NBS to suppress procyclic development. Two lines were prepared from EA44p7 using 3 × 10⁵ metacyclines to initiate the culture (Gray et al., 1984). Mammalian forms maintained at 37°C grew sluggishly, required medium changes at daily intervals and were observed only for 18 days. A line cultured at 28°C produced 5 × 10⁶ trypanosomes/ml and was itself successfully passaged on two occasions. Cultures were also derived from EA44p9 and maintained in MEM-NBS for 70 days. Another line of TREU 1468 (EA31p5) was successfully cultured as mammalian forms



and observed for 57 days. Infectivity of some cultures of TREU 1468 are shown in Table 1.

b) *TREU 1457*. Eight $\times 10^6$ metacyclic trypanosomes from TREU 1457 culture WA22p3 (Gray et al., 1984) were used to initiate a culture on BAE (WA22/BAE). Dividing trypanosomes were observed within 24 h but after 4 days increasing numbers of procyclics were seen. By day 22, procyclics had developed and overgrown the culture. At day 4, 3×10^6 trypanosomes from this primary culture were passaged to two BAE monolayers (WA22p1/BAE). One culture was incubated at 28°C and the other at 37°C. At 37°C, the number of dividing mammalian form trypanosomes decreased until none were present at day 20. At 28°C the mammalian forms increased in number until at day 20 the supernatant contained 1.5×10^6 /ml. Thereafter the presence of intercellular pockets of procyclics made the culture more difficult to maintain. This culture line was successfully passaged three more times at 20 day intervals. In each of these cultures mammalian forms were gradually overgrown by procyclics. Infectivity tests on this line are shown in Table 1.

Culture WA22p2/BAE was observed for 55 days. After it had been passaged to WA22p3/BAE, procyclic trypanosomes were allowed to overgrow the culture, and these further differentiated into epimastigotes. Colonies of epimastigotes could be seen adhering to the flask surface at places where the endothelial cells had been removed. Supernatant containing these epimastigote bundles was passaged into MEM-FCS alone and the trypanosomes behaved in a similar manner to those passaged from cultures containing epimastigotes described in a previous paper (Gray et al., 1984). A complete layer of epimastigote colonies formed on the bottom of the flask from which metacyclic could be harvested by 14 days.

In another culture, initiated with 5×10^6 metacyclic trypanosomes separated from WA22p3 using MEM-NBS, no procyclics appeared by day 60. This culture was passaged at 17 days and thereafter at monthly intervals and retained for over seven months. Infectivity was maintained throughout the period of observation (Table 1).

Biological and morphological characteristics of infective trypanosomes grown on a BAE monolayer

The trypanosomes growing in close association with the cells forming the cell monolayer (Fig. 2) were shown to have intact surface coats (Fig. 3). There

Fig. 2. Scanning electron micrograph of *Trypanosoma congolense* TREU 1457 growing on bovine aorta endothelial cell monolayers. Trypanosomes are attached by their anterior ends (arrowed) ($\times 2315$).

Fig. 3. Transmission electron micrograph of *Trypanosoma congolense* TREU 1457 growing on bovine aorta endothelial cell monolayers ($\times 42,700$). Inset shows the trypanosome surface membrane and compact appearance of the surface coat ($\times 100,000$). E = endothelial cell monolayer; T = trypanosome; S = surface coat.

appeared to be no hemidesmosome attachment points at areas of contact with feeder cell layers. When suspensions of trypanosomes were inoculated into rabbits it was found that lines of TREU 1457 and TREU 1662 cultured in MEM-FCS produced local skin reactions even after 167 days in culture. In contrast, lines maintained in MEM-NBS lost the ability to develop local skin reaction in 13 days, with the exception of a culture of TREU 1468 which still produced skin reactions even after 60 days in vitro. Rabbits infected with *T. congolense* by the bites of *G. morsitans* developed local reactions within seven days of fly bite. Serous exudate from these reactions, containing low numbers of trypanosomes, also produced skin reactions when transferred to other rabbits. Similarly, material taken from these recipient rabbits also retained the capacity to induce local reaction formation when passaged to new hosts.

Discussion

In the presence of eukaryotic cells non-dividing *T. congolense* metacyclic forms derived either from tsetse fly saliva or from in vitro culture transformed to produce cultures of replicating trypanosomes which resembled bloodstream forms found in the mammalian host. This extends the observation by Brun et al. (1981) who observed a similar transformation of *T. brucei* metacyclic forms on fibroblast-like cells. All three stocks of *T. congolense* were established in association with endothelial cells at 28 °C but at 37 °C only cultures of the riverain stock TREU 1662 were readily maintained in vitro. *T. congolense* is known to inhabit the peripheral circulation (Losos and Ikede, 1972) and hence the optimum temperature for culture of mammalian forms of this parasite may be intermediate between the two temperatures used here.

Bovine aorta endothelial cell monolayers prove to be much the best feeder cells for *T. congolense* mammalian forms compared to other bovine cell types, because of the very large numbers of trypanosomes they can support in vitro (M. A. Gray and C. A. Ross, unpublished observations). Whether this is related to the close proximity to *T. congolense* bloodstream forms which these cells have during natural infections is not known.

Cultures initiated with tsetse fly metacyclics and bovine dermal explants were more successful in establishing and maintaining dividing forms than BAE monolayers. Growth factors supplied by these two sources of mammalian material may well be different. One advantage of the dermal collagen explant is that the trypanosomes are locally concentrated during the initial stages, a factor which is also important in the initiation of cultures producing epimastigotes and metacyclic trypanosomes (Gray et al., 1984). The small numbers of *T. congolense* metacyclics available from an infected tsetse fly proboscis or probe makes their local concentration especially desirable. Once cultures had been established with dermal collagen explants, dividing mammalian forms of TREU 1662 were successfully transferred to continuous maintenance on BAE mono-

layers but similar trypanosomes of TREU 1468 did not survive the change in conditions. This variation encountered in the adaptability of the two different *T. congolense* stocks to culture might be related to their zymodeme characteristics, as riverain and savannah stocks, respectively (Young and Godfrey, 1983). In contrast to results obtained with tsetse fly metacyclics, however, cultured metacyclics of both savannah stocks, TREU 1468 and TREU 1457 readily adapted to growth on the BAE monolayer.

One factor affecting the growth of *T. congolense* in vitro on endothelial cells is the nature of serum supplement used in the growth medium. While infective trypanosomes could be grown readily in MEM-NBS, in cultures supplemented with MEM-FCS trypanosomes differentiated into procyclics. This change is similar to the different serum sensitivities observed for different stages of other stocks of *T. congolense* in culture (Brun, 1982).

In MEM-FCS, and in the continued absence of antibiotics, *T. congolense* procyclic forms will differentiate into epimastigotes; from these epimastigotes mature insect form cultures producing infective metacyclic forms can be obtained. Thus cultures which were initiated from metacyclic forms can be manipulated so that first mammalian forms and subsequently all insect forms can be derived. The complete life cycle of *T. congolense* can therefore be grown in vitro, in directly linked but separate culture systems. In addition, each stage can be separated from the other in quantities many times larger than can be obtained in vivo and in conditions which can be more strictly controlled.

When deposited in the skin of rabbits, metacyclic trypanosomes of *T. congolense* invariably induce the development of local skin reactions containing an extravascular focus of parasites: both metacyclics derived from tsetse flies and parasites cultured in vitro are capable of causing these reactions (Luckins and Gray, 1978; Luckins et al., 1981). In contrast, trypanosomes from the bloodstream of the vertebrate host are incapable of causing reactions (Luckins et al., 1981). The ability to develop extravascularly is retained by trypanosomes located within the skin for up to 7 days. Low numbers of such trypanosomes induce local reactions formation in a new host. Extravascular forms of *T. congolense* represent a stage of development intermediate between the metacyclic trypanosomes in the insect vector and bloodstream forms in the mammalian host. Extravascular forms undergo replication, unlike metacyclic trypanosomes, yet they still express metacyclic variable antigens (Luckins and Gray, 1978, 1979). In addition, unlike bloodstream forms, extravascular forms of *T. congolense* retain the ability to cause local skin reactions even when passaged several times through the mammalian host. Trypanosomes grown on endothelial cells in the presence of MEM-NBS and MEM-FCS are morphologically similar to bloodstream forms of trypanosomes from the vertebrate host. However, only trypanosomes cultured in MEM-FCS retain the ability to induce local reactions in rabbits and may represent forms similar to those found in local skin reactions following bites by infected tsetse flies. It is not known why the cultured trypano-

somes differ in this way, but it is possible that in MEM-NBS the population is transformed to a type equivalent to that found in the peripheral circulation of the host.

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