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In vitro cultivation of *Trypanosoma congolense:* the production of infective metacyclic trypanosomes in cultures initiated from cloned stocks

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Summary

Glossina morsitans were infected with two cloned stocks of *T. congolense.* The proboscides, foreguts and midguts of infected flies were then used as sources of trypanosomes in vitro at 28° C in the presence of bovine dermal collagen explants. Cultures were established in which trypanosomes differentiated into adhering colonies of epimastigote forms which could then be maintained and subcultured in Eagle's Minimum Essential Medium supplemented with foetal calf serum for over 40 weeks. Within 2–4 weeks of establishment of each culture or subculture the epimastigote trypanosomes differentiated into metacyclic trypanosomes which could be harvested from supernatant medium at concentrations of 1×10^5–3×10^6 parasites/ml. These organisms were used to induce the formation of local skin reactions in rabbits. Successful cultivation of infective trypanosomes appeared to depend on the initial adhesion of the parasites to the surface of the flask where they subsequently differentiated first into epimastigote and then to metacyclic forms.

Key words: *Trypanosoma congolense;* clone; in vitro cultivation; epimastigotes; metacyclics.

Introduction

The cultivation in vitro of infective forms of *Trypanosoma congolense* from uncloned stocks from different parts of Africa has been described by Gray et al. (1981) and Hirumi et al. (1982). Established cultures of uncloned stocks contain

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epimastigote forms of the parasite which grow as adherent layers on the culture flasks. Epimastigote trypanosomes can be serially passaged in the absence of mammalian or insect cells resulting in long-term cultures which produce metacyclic trypanosomes (Gray et al., 1981).

The present work describes the preparation of cultures from cloned stocks of *T. congolense* from East and West Africa starting with different developmental stages of trypanosomes from the tsetse fly. Cultures were established using different support media and their growth characteristics in primary and serially sub-passaged cultures were studied, including the sequence of morphological differentiation and their infectivity for mammalian hosts. The paper also defines the passage histories and derivation of a number of culture stabilates which have been used in work described here and in subsequent papers.

Materials and Methods

Trypanosomes

Trypanosomes used to initiate cultures were obtained from *Glossina morsitans* 43–83 days after infection with either *T. congolense* TREU 1457, a cloned derivative of TREU 1290, or *T. congolense* TREU 1468, a cloned derivative of TREU 1173 (Luckins et al., 1983). The sources of the stocks and the method of infection of *G. morsitans* are described by Gray et al. (1981).

Establishment of cultures and maintenance of culture lines

Primary cultures were initiated from infected tsetse proboscides in culture flasks containing Eagle's Minimum Essential Medium supplemented with 20% foetal calf serum (FCS), plus 2 mM glutamine (designated MEM) and dermal collagen explants as described by Gray et al. (1981). Similar cultures were started in RPMI 1640 and M199, also supplemented with 20% FCS and 2 mM glutamine. All media and sera were obtained from Gibco Europe LTD: No antibiotics were used in any media later than six days after initiation of primary cultures, since it was found that they had a deleterious effect on the appearance of epimastigotes (Gray and Luckins, 1982).

Attempts were also made to initiate cultures from trypanosomes in the foregut, including the proventriculus, and the midgut of infected tsetse flies. The gut was removed from the thoracic cavity using sterile dissecting forceps after decapitation of the fly and removal of the abdomen from the thorax. A portion of the foregut containing numerous proventricular trypanosomes or midgut containing procyclic forms was placed beside dermal explants in culture vessels containing MEM. In one culture the foregut from a fly infected with TREU 1457 was placed in a flask which contained MEM but no explant. Cultures were designated with the number of the tsetse fly from which they were established, and the two stocks were identified by the prefix WA and EA for TREU 1457 and 1468 respectively.

Subcultures were first made when an adhering layer of epimastigotes was formed. After the first passage, cultures were serially passaged at approximately monthly intervals by transferring supernatant containing 1×10^7 trypanosomes into a fresh flask without dermal explant and adding fresh medium up to a volume of 4 ml.

Assessment of growth characteristics of cultures

Cultures were examined for trypanosome growth and infectivity as described by Gray et al. (1981). However, the staining technique in morphological examinations was based on that of Robinow (1944) for demonstrating chromatin material in bacteria. Thin smears were air dried, fixed in methanol, and then hydrolysed in NHCl at 60° C for 9 min. They were washed in phosphate buffer

pH 7.0, stained in 5% Giemsa (Gurr's Improved R66 solution, Hopkins and Williams) in phosphate buffer for 65 min at 37° C and then rinsed in the buffer and blotted dry before examination.

Cryopreservation and re-establishment of cultures

Culture supernatants containing parasites were cryopreserved in 2.0 ml glass vials using 7.5% glycerol as described by Gray et al. (1981). In cultures where only a few epimastigotes were present in the supernatant the organisms were concentrated by centrifugation for 10 min at 500 g, after which they were resuspended in 2 ml medium containing 7.5% glycerol. Cultures were re-established by thawing a vial, placing the material in a culture flask, adding an equal amount of medium and then gassing with 5% CO_2 and incubating at 28° C. Complete medium changes were made at 2-day intervals.

Separation of metacyclic trypanosomes

Separation of metacyclic forms of trypanosomes in culture was done using diethylaminoethyl cellulose columns (Whatman DE52, Whatman Lab Sales) equilibrated with phosphate buffered saline pH 8.1, containing 1% w/v glucose (PSG). DE52 columns were prepared in the barrels of 10 ml plastic syringes using 2 mm of packed glasswool for support. Nine ml of DE52 were placed in the syringe and 4–8 ml of culture supernatant containing the trypanosomes were poured into the column. Ten ml of PSG were used to elute the infective trypanosomes from the DE52. These procedures were carried out aseptically so that trypanosomes could be re-established in culture when required.

Infectivity tests

Primary cultures were monitored every 8 to 14 days for the acquisition of infective trypanosomes by injecting culture supernatants intraperitoneally into mice.

The numbers of infective organisms in cultures were estimated using the technique described by Gray et al. (1981). However, since the absolute number of infective forms produced was influenced by different batches of media or FCS, titrations were carried out using parasites which had been eluted from DE52 columns to determine the proportion of infective parasites present in the metacyclic population. In addition, New Zealand White rabbits were inoculated with 1×10^5 DE52 column-seperated trypanosomes to determine the ability of infective trypanosomes to induce the formation of local skin reactions (Luckins et al. 1982).

Results

Initiation of cultures of TREU 1457

1. From an infected proboscis with bovine dermal collagen explant

a) *In MEM.* – Five cultures were intiated each from a single infected proboscis. Two cultures, WA44 and WA56, showed low numbers of epimastigote colonies adhering to the flask at 10 weeks and by 15 weeks and 12 weeks respectively epimastigotes had spread over the whole of the flask. WA56 produced infective trypanosomes after 13 weeks and WA44 by 20 weeks. The remaining three cultures were discarded due to contamination or non-appearance of trypanosomes.

Culture WA56 was passaged at 11 weeks and WA44 at 13 weeks and both subcultures produced metacyclic trypanosomes within 5 weeks. Both subcultures were successfully cryopreserved.

Table 1. Derivation and designation of lines of *Trypanosoma congolense* TREU 1457 cultured in vitro

Number of infective fly	Primary culture line	Designation of sub-passaged cultures and day of passage	Cryopreserved on days	Day on which infectivity to mice acquired
WA21 (proboscis)	1457–1	$WA21_{p1}$, 54	46; 51; 107	50
		$WA21_{p2}$, 37	25; 27; 31; 72	14
		$WA21_{p3}$, 50		NT
		Total number of days in vitro = 148		
WA 30 (proboscis)	1457–2	$WA30_{p1}$, 38	48; 52; 56	28
		$WA30_{p2}$, 36	25; 31	14
		$WA30_{p3}$, 32	39	16
		$WA30_{p4}$, 36		14
		$WA30_{p5}$, 34		14
		$WA30_{p6}$, 29	28; 30; 36	NT
		$WA30_{p7}$, 28		28
		$WA30_{p8}$, 31		NT
		Total number of days in vitro = 292		
WA22 (foregut)	1457–3	$WA22_{p1}$, 88d*		21
		$WA22_{p2}$, 49	19	NT
		$WA22_{p3}$, 17	–	NT
		$WA22_{p4}$, 18	14	14
		Total number of days in vitro = 186		
WA24 (pro-ventriculus)	1457–4	$WA24_{p1}$ 59		NT
		$WA24_{p2}$ 38		NT
		$WA24_{p3}$ 22		7
		$WA24_{p4}$ 10		NT

* Derived from cryopreserved primary culture
NT = not tested

b) *In M199.* – Four cultures, WA7, WA21, WA28 and WA30 developed sparse patches of adhering epimastigotes by 4 weeks but infective trypanosomes were produced from two weeks and were present until 8 weeks after initiation. After 10 weeks adhering epimastigotes were not seen and the cultures were no longer infective.

Cultures WA21 and WA30 were passaged at 8 weeks into both M199 and MEM. All subcultures gave adhering layers of epimastigotes over the bottom of the flask and produced infective forms within 2–5 weeks of sub-passage. In M199 the adhering epimastigote colonies were gradually lost and metacyclics could not be separated on DE52 columns. Only the lines in MEM, 1457–1 and 1457–2 were serially passaged (Table 1). The numbers of metacyclic trypanosomes collected at media changes was approximately $1–2 \times 10^6$ per ml giving $4–8 \times 10^6$ per flask.

2. From infected foregut in MEM with a bovine dermal collagen explant

Two cultures, WA35 and WA22 were initiated from trypanosomes in the foregut of tsetse flies. A layer of epimastigotes was present in culture WA35 by 3 weeks and infective forms appeared by 4 weeks. In culture WA22 an adhering layer of epimastigotes was present by 6 weeks and infective forms by 10 weeks. This culture was cryopreserved at 12 weeks, resuscitated in vitro after one month and designated 1457–3 (Table 1). On subculture it produced infective forms after 3 weeks and was thereafter serially passaged on 4 occasions for a total of 32 weeks in vitro.

3. From an infected foregut in MEM alone

In culture WA24 colonies of adhering epimastigotes appeared by 6 weeks but no metacyclics were produced during an 11-week observation period. The culture was passaged at 7 weeks. Epimastigotes established as an adhering layer in the subculture and metacyclic trypanosomes were produced after 7 weeks. This line was serially passaged and designated 1457–4 (Table 1).

4. From a midgut in MEM with a bovine dermal collagen explant

In culture WA22a, initiated from a midgut, short blunt-ended epimastigotes adhering to the surface of flask were seen by 7 weeks. These increased in length by 10 weeks at which time the culture became infective.

Initiation of cultures of TREU 1468

1. From an infected proboscis with bovine dermal skin explant

a) *In MEM.* – Seven cultures were each initiated from different tsetse proboscides. Culture EA39 showed a few dividing trypanosomes in close association with fibroblast cells originating from the dermal skin explant. The trypanosomes were morphologically similar to trypanosomes found in the bloodstream of mammalian hosts and were infective for mice. Infective forms were produced for 12 weeks before the culture was discarded as no adhering bundles of epimastigotes developed. Although cultures remained infective for this period most of the trypanosomes had differentiated into procyclic forms after 4 weeks.

Culture EA44 was also infective for mice from 3 weeks after establishment although the supernatant showed mainly proventricular forms. This culture continued to produce infective forms for the next 17 weeks. Adhering bundles of epimastigotes on the flask surface were seen at 12 weeks which spread to cover 25% of the surface over the next 4 weeks. The culture was passaged at 90 and 98 days and supernatants from the two subcultures were pooled at D26 and D18 respectively, concentrated by centrifugation and cryopreserved. When resuscitated after 3 weeks, adhering epimastigotes developed which covered the flask by 6 weeks and differentiated into infective metacyclic trypanosomes by

8 weeks. This culture line, 1468–1, was serially passaged for a total of 10 months and produced an average of $1–3 \times 10^5$ metacyclic trypanosomes/ml at each medium change (Table 1). The five remaining cultures failed to give adhering epimastigotes and did not produce infective trypanosomes within a 10 week observation period.

b) *In M199.* – Epimastigotes failed to develop in five cultures during a 10-week observation period, although in two cultures trypanosomes infective for mice were present for the first 4 weeks.

c) *In RPMI.* – Epimastigotes failed to develop in five cultures during a 10-week observation period but in one, EA13, the trypanosomes morphologically resembled bloodstream forms. They were present in close association with fibroblasts growing from the skin explant and were seen between 17 and 28 days after initiation, then gradually differentiated into procyclic forms. This culture was infective for mice for 10 weeks.

2. From an infected foregut in MEM with a bovine dermal collagen explant

In culture EA31 infective forms were present from four weeks onwards although no obvious adhering bundles of epimastigotes were seen for 13 weeks. Thereafter, epimastigotes spread to cover 25% of the flask. The culture was cryopreserved at 116 and 118 days, after concentrating the trypanosomes by centrifugation. When reconstituted after 3 weeks few adhering epimastigotes were present but numbers increased until by 18 weeks epimatigotes formed an adherent layer covering the surface of the flask. Metacyclics were first detected at 20 weeks and this culture was serially passaged as line 1468–2 from 18 weeks for a total of 56 weeks, producing an average $1 \times 10^5–3 \times 10^5$ metacyclic trypanosomes/ml in the supernatant of each medium change (Table 2).

Observations on morphology and differentiation

a) Primary cultures of TREU 1457 and TREU 1468 derived from a proboscis

In cultures initiated from proboscides several free proventricular trypanosomes left the proboscis within a few hours and then adhered and multiplied in the vicinity of the dermal explant. These forms shortened and atypical forms gradually appeared, apparently arising as a result of incomplete division since they possessed several nuclei and kinetoplasts. Trypanosomes free in the supernatant continued to resemble proventricular forms. The adherent colonies of atypical organisms were replaced by colonies of short trypanosomes with pointed ends. These adherent trypanosomes differentiated into blunt-ended epimastigotes about 7 μm in length and individual epimastigotes also began to appear in the supernatant. The attached epimastigotes increased in length to 10–20 μm, and their numbers increased. This resulted in bundles of epimastigotes, attached by their anterior ends, spread over the surface of the flask, eventually covering the entire area and also appearing in the supernatant. Two to three

Table 2. Derivation and designation of lines of *Trypanosoma congolense* TREU 1468 cultured in vitro

Number of infective fly	Primary culture line	Designation of sub-passaged cultures and day of passage	Cryopreserved on days	Day on which infectivity to mice acquired
EA44	1468–1		118/120	
(proboscis)		$EA44_{p1}$, 118*	(26 + 18) (28 + 20)**	
		$EA44_{p2}$, (28 + 20)		58
		$EA44_{p3a}$, 28		NT
		$EA44_{p3b}$, 48	24	22
		$EA44_{p4a}$, 51	19	NT
		$EA44_{p4b}$, 22		18
		$EA44_{p4a}1$, 62		NT
		$EA44_{p5a}1$, 16		16
		$EA44_{p6a}1$, 31		14
		$EA44_{p7a}1$, 18		16
		$EA44_{p8a}1$, 30		NT
		$EA44_{p9a}1$, 16		16
		Total number of days in vitro = 349		
EA31	1468–2		118/20	28
(foregut)		$EA31_{p1}$, 118*	141	121
		$EA31_{p2}$, 121	18	20
		$EA31_{p3}$, 16		12
		$EA31_{p4}$, 32		NT
		$EA31_{p5}$, 18	43	16
		$EA31_{p6}$, 25		NT
		Total number of days in vitro = 369		

* Derived from cryopreserved primary culture
** Material from two lines of sub-passage $EA44_{p1}$ pooled on days shown
NT = not tested

weeks after this stage had been reached short forms morphologically resembling metacyclic trypanosomes were seen within epimastigotes clusters. Supernatants from mature cultures contained a total of $1–5 \times 10^7$ organisms per ml, predominantly bundles of epimastigotes. In some primary cultures containing a dermal explant dividing trypanosomes, morphologically similar to bloodstream forms, were also present.

b) Primary cultures of TREU 1457 and TREU 1468 initiated from foregut or midgut

Many proventricular forms emerged from the proventriculus and the midgut (1×10^5/ml) and adhered to the surface of the flask especially in the area of the dermal collagen explant. They shortened to forms with small pointed ends in bundles attached by their anterior ends and thereafter gradually devel-

Table 3. Infectivity titrations in mice of metacyclic forms of *Trypanosoma congolense* cultured in vitro

Culture designation	Numbers of metacyclic trypanosomes (log_{10}/ml)	Numbers of infective metacyclic trypanosomes (log_{10} ID_{63}/ml)
$WA30_{p3}$	5.2	3.8 ± 0.3
$WA30_{p6}$	5.2	4.0 ± 0.3
$WA21_{p1}$	5.2	3.1 ± 0.3
$EA44_{p8(1)}$	5.0	3.1 ± 0.5
$EA44_{p8(2)}$	5.9	3.1 ± 0.5
$EA44_{p9}$	5.5	3.0 ± 0.5

oped into colonies of long epimastigotes as in cultures initiated from infective proboscides.

c) Serially passaged cultures TREU 1457 and TREU 1468

Long epimastigotes were predominant in cultures at the time of passage. Most attached immediately to the new flask and within 24–28 h shortened considerably. In some the nucleus appeared to move behind the kinetoplast and the trypanosomes resembled proventricular forms. The supernatant contained free epimastigotes and metacyclics which were depleted at the first medium change. During the next 2–6 days the adherent trypanosomes changed back to short blunt-ended epimastigotes whilst the supernatant contained predominantly proventricular forms. After 8 days the attached epimastigotes had lengthened and increased numbers were seen in the supernatant. Two weeks after passage the cultures contained an adherent layer of clusters of long epimastigotes in some of which metacyclic trypanosomes could be seen attached by their anterior ends. The supernatant contained bundles of epimastigotes and free metacyclic forms. Once the adhering layer of long epimastigotes was established the culture could be passaged with ease but, unlike primary cultures, no dividing bloodstream trypanosomes were seen.

Infectivity tests

Representative examples of the results of infectivity titrations are shown in Table 3. Little difference was seen in the number of infective organisms produced by different lines of different stocks but there was some variation in the proportion of the infective trypanosomes present in the whole metacyclic population.

Column-separated trypanosomes inoculated into rabbits caused local reactions even after considerable periods in culture. For instance, in line 1457–2 passage WA_{p3} caused local skin reactions at 160 days, in 1457–3 passage

$WA22_{p4}$ produced reactions at 168 days and in line 1468–2, $EA31_{p2}$ caused local skin reactions after 251 days in culture.

Discussion

The successful establishment in vitro of two different cloned stocks of *T. congolense* from East and West Africa, producing parasites capable of infecting the vertebrate host, has confirmed earlier studies using an uncloned West African stock (Gray et al. 1981). The main criterion for successful culture appeared to be initial adhesion of organisms to the culture vessel enabling differentiation into epimastigotes to occur as suggested by Hommel and Robertson (1976). Although differentiation into epimastigotes seems to require adherence of trypanosomes to the culture flask the two events can be widely separated in time. The differentiation of the attached forms to epimastigote can take several weeks, but once epimastigote colonies appear their subsequent proliferation seems to be enhanced. This property may be similar to the observation of epimastigote behaviour in the labrum of infected tsetse flies where epimastigotes grouped in compact colonies and in close proximity to other individuals stimulated hemidesmosome attachment points between flagella and cuticle (Thévenaz and Hecker, 1980). These authors also showed that trypanosomes attached to the labrum possessed surface coats, suggesting that differentiation to metacyclic trypanosomes occurs whilst the organisms are still attached (Thévenaz and Hecker, 1980). In established cultures of *T. congolense* adherent clusters of epimastigotes also contained forms morphologically similar to metacyclic trypanosomes.

The morphology and differentiation of *T. congolense* in vitro follows a very similar pattern to that observed in vivo in the tsetse fly (Lloyd and Johnson, 1924; Thévenaz and Hecker, 1980). The proboscis of an infected fly contains free proventricular forms, attached epimastigotes and metacyclic trypanosomes. Probably the free proventricular forms pass out of the labrum, adhere initially and multiply around the dermal collagen explant, rather than the epimastigotes which are firmly attached to the proboscis. Low numbers of metacyclic trypanosomes are also free and may pass into the medium and it appears that, in the presence of skin tissue from the natural host, these differentiate and divide as infective forms. Since the proboscis is placed in close proximity to the dermal explant the released trypanosomes are thereby locally concentrated.

This localisation of *T. congolense* may be an important function of the dermal explant or the explant might be supplying growth factors. The increased time taken for culture WA6 to reach maturation in the absence of dermal explant may indicate the need for adherence or growth factors supplied by such explants.

Cultures started from trypanosomes in the isolated foregut preparations also matured to infectivity indicating that neither epimastigote nor metacyclic trypanosomes are essential to the initiation of the differentiation process. Instead, the greater numbers of proventricular forms available in these preparations (EA31, WA22, and WA35) may have allowed their more rapid differentiation to cultures producing infective forms.

The establishment of predominantly epimastigote cultures which produce metacyclic trypanosomes can thus be initiated from trypanosomes at several stages of the life cycle of *T. congolense* in the tsetse fly. We have also shown that cultures can be initiated from bloodstream forms of these stocks (Ross, unpublished observations) and Hirumi et al. (1982) have described similar experiments with other stocks of *T. congolense*.

Since MEM has proved to be a suitable medium for establishment of adherent layers of epimastigotes from uncloned stocks of *T. congolense* it was also used here in the present work. In contrast, when using RPMI 1640 and M199, no adherent epimastigote layers were found in cultures derived from TREU 1468 during the 10-week observation period. M199 did support differentiation of *T. congolense* but it was thought that the presence of detergents interfered with the attachment of epimastigotes and chromatographic separation of metacyclics. Trial cultures in this medium were therefore adapted to MEM which is the medium of choice in this laboratory. However, samples of MEM more than 4 months old consistently produced much lower numbers of metacyclics. It was also found necessary to test individual batches of FCS since considerable variation occured in the numbers of metacyclics produced in vitro using particular sera. Sera which supported growth of epimastigotes and production of 1×10^6 metacyclics per ml were selected for use and stored at $-20°$ C for periods up to 2 years. The two stocks initially showed considerable differences in the numbers of metacyclics produced in culture, suggesting that their growth requirements in vitro were different and that TREU 1457 was better able to adapt to MEM than TREU 1468.

In two cultures, EA13 and EA39, infective trypanosomes were produced soon after initiation in the absence of epimastigotes. It is probable that this could be accounted for by the differentiation of metacyclics into mammalian forms in the presence of cells of the dermal explant.Brun et al. (1979) showed that metacyclic trypanosomes of *T. brucei* differentiate and multiply in the presence of mammalian cells. In *T. congolense* cultures where there was early acqusition of infectivity the trypanosomes differentiated into procyclic forms which accounts for the eventual loss of infectivity of the cultures.

Infectivity tests showed that only a small proportion of the cultured metacyclics were infective for mice. Possibly some of the organisms were immature forms which did not have a complete surface coat, the washing procedure on the chromatography columns damaged the coat, or intraperitoneal injection was hazardous for metacyclic trypanosomes.

The ability to culture in vitro large numbers of metacyclic trypanosomes of cloned stocks of *T. congolense* has important consequences for furthering our understanding of antigenic variation during this stage of parasitic development. The extent of the antigenic heterogeneity of metacyclic variable antigen types (VATs) may be more readily determined in cultures than in tsetse flies in which only low numbers of parasites are available for analysis. Little work has been done on biochemistry of the variable antigens of metacyclic trypanosomes and these cultures could also provide a soucre of large amounts of material for analysis. The development of monoclonal antibodies to metacyclic VATs of *T. congolense* using our culture system has already been achieved (Crowe et al., 1983), and it is expected that their use in biochemical studies will allow greater definition of the surface antigens.

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