Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 45 (1988)
Heft: 1

Artikel: Cultivation of the life cycle stages of "Trypanosoma brucei" sspp.
Autor: Kaminsky, R. / Beaudoin, E. / Cunningham, I.
DOI: https://doi.org/10.5169/seals-314056

Acta Tropica 45, 33–43 (1988)

Center for Parasitology, Zoology Department, University of Massachusetts, Amherst, Massachusetts 01003, USA

Cultivation of the life cycle stages of *Trypanosoma brucei* sspp.

R. KAMINSKY[1], E. BEAUDOIN, I. CUNNINGHAM

Summary

A culture system was devised for the production of the various stages in the developmental cycle of *Trypanosoma brucei brucei* and *T. b. rhodesiense.* The bloodstream forms were grown at 37° C on a feeder layer of fibroblasts from embryos of *Microtus montanus* or CD-1 mice in HEPES-buffered Minimum Essential Medium with Earle's salts, supplemented with 15% heat-inactivated rabbit serum. When they were transferred to HEPES-buffered Cunningham's medium and incubated at 27° C, they transformed into procyclic trypomastigotes, some of which developed into epimastigote and metacyclic forms in the presence of explants of *Glossina morsitans* or *Phormia regina.* Stages infective to mice were produced in cultures of procyclic forms grown with *Anopheles gambiae* cells in medium consisting of a mixture of 3 volumes of Cunningham's medium and 1 volume of *Anopheles* cell medium. In vitro-produced metacyclic trypanosomes were used to initiate cultures of bloodstream forms.

Key words: *Trypanosoma brucei brucei; T. b. rhodesiense;* in vitro cultivation; life cycle.

Introduction

During the past decade there have been significant advances in the cultivation of the various life cycle stages of African trypanosomes. The early workers using a monophasic medium with nutrient agar (Novy and MacNeal, 1904) or liquid (Pittam, 1970) and biphasic (Tobie, 1964) media containing blood were able to grow only the procyclic forms which are similar to those in

[1] Present address: ILRAD, P.O. Box 30709, Nairobi, Kenya

Correspondence: Dr. Isabel Cunningham, Department of Zoology, University of Massachusetts, Amherst, Massachusetts 01003–0027, USA

the midgut and proventriculus of a tsetse fly. However, successful cultivation of bloodstream forms (Hirumi et al., 1977; Hill et al., 1978; Brun et al., 1981; Balber, 1983) and metacyclic stages (Cunningham and Honigberg, 1977; Cunningham et al., 1981; Jones et al., 1981; Cunningham, 1986; Cunningham and Kaminsky, 1986) of *Trypanosoma brucei* sspp. has resulted from the use of liquid media in organ or cell cultures. In this report we describe a system for the cultivation of the complete developmental cycle of *Trypanosoma b. brucei* and *T. b. rhodesiense.*

Materials and Methods

History of the trypanosome stocks

Trypanosoma b. brucei TRUM (Trypanosome Research University of Massachusetts) 397 is a population of procyclic forms cultivated at 27° C for 33 days in the medium of Cunningham (1977), containing 25 mH HEPES and 20% foetal bovine serum (referred to as SMH). It was derived from TREU 1194 which was obtained from a cyclically transmitted population of TREU (Trypanosome Research Edinburgh University) 667 (EVE 10) isolated by the Edinburgh Veterinary Expedition to the Busoga district of Uganda in 1966 (Reid et al., 1970). This stock can be cyclically transmitted and causes relapsing and chronic infections in mice.

T. b. rhodesiense TRUM 589 consists of procyclic forms established from a 4-day infection of bloodstream forms from a CD-1 mouse infected with EATRO (East African Trypanosome Research Organization) 1895. This latter population had been isolated from a naturally infected male patient from Lugala, Uganda, in 1971. Trypanosomes from a mouse 7-day post-inoculation with in vitro-produced metacyclic forms were used to initiate cultures of bloodstream forms. Thereafter the organisms underwent two developmental cycles in vitro (see Results) and the resulting population of procyclic forms was cryopreserved as TRUM 601. This stock is cyclically transmissible and causes relapsing and fatal infections in mice.

Preparations and maintenance of the cultures

Cell cultures: Monolayers of feeder cells were prepared by treatment of muscle tissue from 10–12-day-old embryos of *Microtus montanus* or CD-1 mice with 0.5% trypsin in Ca^{++} and Mg^{++}-free Earle's salt solution for 10 min at 37° C. About one ml calf serum was added to arrest trypsin action and the tissues were triturated with a wide-tipped Pasteur pipette. The suspension was centrifuged at 900 *g* for one min and the supernatant containing the cells was spun at 250 *g* for 10 min. The supernatant fluid was discarded and the cells were resuspended in MEMF (Brun et al., 1981) containing 10% calf serum. The cells were then dispensed in 4 ml aliquots into 25 cm^2 Nunclon culture flasks (henceforth referred to as flasks) at a concentration of $\sim 2 \times 10^6$ cells/ml and incubated at 37° C in an atmosphere of 5% CO^2 in air. When a culture developed into a confluent monolayer it was treated with TV (Trypsin-Versene) solution and the cells were subpassaged. This solution was prepared by dissolving NaCl 8.00 g; KCl 0.40 g; Glucose 1.00 g; $NaHCO_3$ 0.58 g; Trypsin solution (Difco 1:250) 0.50 g; EDTA disodium salt 0.20 g and 2 ml Phenol red (0.5% sol.) in 1 litre of distilled water. The mixture was filter sterilized and used as follows:

Method 1. – Remove medium from the flask and add 2 ml warm TV solution. Remove the supernatant fluid when the cells are seen to round up. Leave the cells in the remaining (~0.3 ml) TV solution for a few more minutes and add 4 ml medium. Disperse the cells and distribute into other flasks e.g. 1:3 split.

Method 2. – After removing the medium rinse the monolayer twice with warm TV solution. Remove all but 0.3 ml TV solution and leave the culture for 5–10 min at room temperature. Add 4 ml medium, disperse the cells and subculture as above. The cultures were subpassaged for up to 15 occasions without any noticeable deterioration of the cells. Populations from the 3rd or 4th passage were cryopreserved in medium containing 10% DMSO for future use.

The cultures of *Anopheles gambiae* cells were established from a sample obtained from Dr. Imogene Schneider, Walter Reed Army Institute of Research, Washington. They were grown at 27° C in flasks in Kitamura's Medium modified by Varma and Pudney (1969) containing 15% foetal bovine serum. When the cells formed a thick layer they were subpassaged by flushing with medium using a Pasteur pipette. All but 0.25–0.5 ml cell suspension was removed and replaced with 3.5–4.0 ml fresh medium.

Aseptic rearing of Phormia regina Meigen

It was necessary to have a source of sterile material and *Phormia regina* was reared aseptically by the method of Dethier and Goldrich (1971). The eggs were sterilized within 24 h after oviposition by being soaked in 1% NaOH for 10 min and rinsed twice in 70% ethanol. They were then placed on a sterile medium (66.7 g powdered yeast, 66.7 g powdered whole milk, 2.83 g Tegosept and 13.3 g agar dissolved by boiling in 1 litre distilled water, autoclaved in sterile flasks for 7 min at 121° C and allowed to cool) and incubated at 27° C. After 10–12 days, when the larvae were fully developed and formed clusters around the cotton wool plug of the flask, they were placed in a filter funnel with filter paper moistened with 70% ethanol. They were rinsed twice in 70% ethanol, twice in sterile distilled water and put into a flask containing sterile wood shavings and placed in the incubator. The larvae pupated within 24 h and adult flies began to emerge after one week. They were removed by chilling for a few min at –18° C or by allowing them to enter an empty conical flask held over the mouth of their flask. The puparia containing flies which had not emerged were used for the tissue cultures.

Trypanosoma cultures

Bloodstream forms. The cultures of bloodstream forms initiated either from infected mouse blood or from in vitro-produced metacyclic trypanosomes were grown in *Mictrotus montanus* or mouse embryo cell cultures at 37° C in 4 ml MEMB (Brun et al., 1981) with 15% freshly prepared heat-inactivated rabbit serum. They were incubated in a 5% CO_2 in air atmosphere and maintained by removing half of the medium and replacing it with an equal volume of fresh medium every second day during the first 7–10 days. Thereafter all but 1 ml of culture fluid was removed and replaced with 3 ml fresh medium daily.

Vector stages

Procyclic forms: Cultures were prepared either from infected mouse blood by the method of Cunningham (1977), the medium being modified by the addition of 25 mM HEPES to form SMH medium, or from bloodstream forms grown in vitro. In the latter case, about 3 ml trypanosome suspension was centrifuged at 900 *g* for 5 min. The supernatant fluid, except for 0.25 ml, was discarded and the pellet was resuspended and mixed with 0.5 ml SMH medium. The suspension was then placed in a flask and incubated at 27° C. About one ml medium was added every second day until the flask contained 4 ml. When the population reached 2×10^7 trypanosomes/ml the culture was subpassaged into 3 flasks and they were maintained by removing all but 0.5 ml suspension and adding 3.5 ml SMH medium every 2 days.

Tsetse fly-trypanosome cultures: The tsetse fly tissue and trypanosome cultures were prepared by the method of Cunningham et al. (1981) and Cunningham (1986) but medium SMH was used. Explants referred to as "abdomen" included the tissues of the alimentary tract. Samples of procyclic trypanosomes were placed in flasks containing 3.5 ml medium and explants freshly dissected from pharate *Glossina morsitans* immediately before emergence. The initial inoculum was $3–4\times10^6$ trypanosomes/ml. The cultures were incubated at 27° C and maintained by removing all except 0.5 ml of trypanosome suspension and adding an equal volume (~3.5 ml) of medium every 2 days. Antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) were included in the medium only during the first week of the culture period.

Phormia-trypanosome cultures: These cultures were prepared and maintained by the method used for the tsetse fly tissue cultures.

Anopheles cells-trypanosome cultures: When the cells formed a monolayer they were incubated for 2 days in medium consisting of 3 vol. SMH and 1 vol. *Anopheles* medium with 20% heat-inac-

tivated foetal bovine serum. Procyclic forms were added to cultures containing 3.5-4.0 ml of this medium to give a concentration of 5×10^6/ml. During the following 5 or 6 days about half of the medium was removed and replaced with fresh medium daily. When trypanosomes were seen attached to the cells, all the trypanosome suspension was replaced with fresh medium daily or occasionally every second day.

Inoculation of mice

In most cases trypanosomes from the cultures were injected into separate CD-1 mice 3 times weekly, but sometimes only once or twice a week. About 3 ml trypanosome suspension from individual flasks were centrifuged at ~1000 *g* for 10 min. All but 0.5 ml of the suspension fluid was discarded. The pellet of organisms was resuspended in the medium in the tube and injected into 5–9-week-old mice. A drop of their tail blood was examined for 30 days for the presence of trypanosomes.

Infectivity titration

The number of infective trypanosomes in individual cultures was estimated by the method of Lumsden et al. (1963). Trypanosome suspensions grown for 1 or 2 days after addition of fresh medium to the cultures were diluted serially ten-fold with medium, and 0.1 ml aliquots were inoculated into groups of 6 mice. The number of trypanosomes in the original suspension was counted in a Neubauer haemocytometer.

Collection of metacyclic trypanosomes

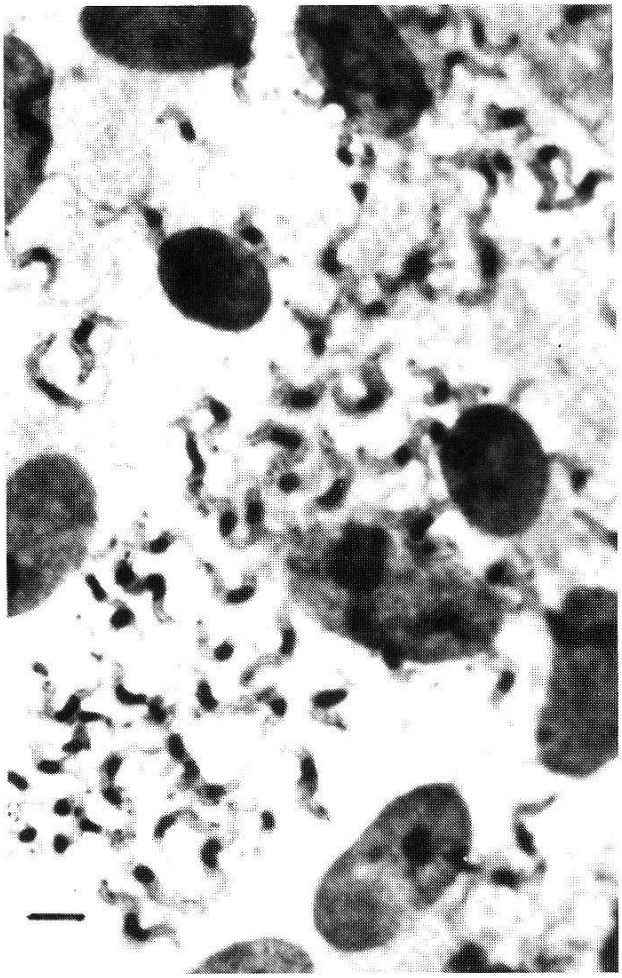
When 2 consecutive inoculations of trypanosome suspensions from a culture incubated at 27° C produced infections in mice, the culture was considered to be infective. The metacyclic forms were harvested by the method of Gardiner et al. (1980) using DEAE-cellulose columns. A 20×1 cm glass chromatography column containing Whatman No 41 filter paper was packed to a height of 7–9 cm with DE-52 equilibrated to pH 8.0 with phosphate saline glucose (PSG) (Lanham and Godfrey, 1970). About 3.5 ml trypanosome suspension was applied to the column and when trypanosomes were observed in drops of eluate, 12 ml were collected into centrifuge tubes using PSG. The eluate was centrifuged at 900 *g* for 15 min. All but 0.2 ml supernatant fluid was discarded and the metacyclics were resuspended in the PSG left in the tube and were used to initiate cultures of bloodstream forms or were processed for microscopy.

Results

Trypanosoma b. rhodesiense

Cultures of bloodstream forms of TRUMs 589 and 601 were prepared by the method of Brun et al. (1981) using blood from the tail of a 7-day infected mouse. The trypanosomes grew in a biphasic condition with the long slender forms in clusters (Fig. 1) closely associated with the cells and the intermediate and short stumpy organisms (Fig. 2) in the liquid phase of the cultures. Mice inoculated with culture trypanosomes on the 7th and 11th days developed relapsing infections after 3 days and died about 1 month later.

Bloodstream forms from a 12-day-old culture were used to initiate cultures of procyclic forms (Fig. 3). After 3 or 4 days they had transformed into procyclic trypanosomes and were not infective to mice. On the 11th day samples were placed in cultures containing explants of *Glossina morsitans*. As shown in Table 1, the flask containing 6 abdomens began to produce infective trypanosomes after 12 days and infectivity persisted for a further 30 days when inoculations of



1

3

2

4

5

Figs. 1–5. All figures of *T. b. rhodesiense.* 1. Bloodstream forms on a monolayer of *Microtus* cells. 2. Bloodstream forms in the liquid phase of *Microtus* cell cultures. 3. Procyclic forms. 4. Epimastigote form. 5. Metacyclic trypanosomes in a 30 days old *Glossina* tissue culture. – Scale = 10 μm. Giemsa stain.

Table 1. Infectivity reacquisition of *Trypanosoma b. rhodesiense*[a] TRUM 589 cultivated at 27°C in medium SMH containing tissue explants of *Glossina morsitans*

No. of explants per flask	Days with explants before infectivity restoration	Period of infectivity (days)	Infections in mice	
			No infected / No inoculated	Prepatent period (days)
13 Hds+SG[b]	18	24[d]	6 / 7	5–9
6 Abdom[c]	12	30[d]	7 / 7	5–9
None	18	9	2 / 7	9–15

[a] Trypanosomes grown for 12 days before added to cultures
[b] Hds+SG = Heads with salivary glands
[c] Abdom = Abdomen and alimentary tract
[d] Experiment terminated

mice were discontinued. The culture with head-salivary gland tissues became infective on the 18th day. The prepatent periods in the mice infected with trypanosomes grown in *Glossina* explants ranged from 5–9 days. The organisms in the control culture without tissues gave rise to sporadic infections with longer prepatent periods in the mice.

Metacyclic forms from a 25-day-old *Glossina* abdomen culture were added to a monolayer of *Microtus* cells and within one day had transformed into bloodstream forms. Mice inoculated with culture samples on days 7, 12 and 22 developed relapsing infections. The pattern of growth of these cultures was similar to the cultures prepared from infected mouse blood and were terminated after 52 days. As indicated in Table 3, it was estimated that there were 5.4×10^4 infective trypanosomes/ml in a population of 6.9×10^5/ml in the supernatant medium of a 21-day-old culture.

On days 8, 12 and 22 trypanosomes from a bloodstream form culture transferred to medium SMH and incubated at 27°C became non-infective procyclic forms after 3 or 4 days. The procyclics derived from the 8-day bloodstream form sample were grown for 16 days and cryopreserved as TRUM 601.

Trypanosomes from the stabilate TRUM 601 were cultivated in SMH for 6 days and then added to flasks containing *Anopheles gambiae* cells. Metacyclic forms were produced after 12 days and the culture remained infective for 63 days when it was terminated (Table 2). The prepatent periods in the mice varied from 1–7 days and were followed by relapsing but fatal parasitaemias. Trypanosomes from the control culture without cells were not infective. An infectivity titration carried out on a 27-day-old culture revealed that there were more than $2.5\pm0.5\times10^5$/ml metacyclics in a total population of 3.3×10^6/ml. Giemsa stained smears of infective cultures contained epimastigote (Fig. 4) and metacyclic (Fig. 5) forms.

Trypanosoma b. brucei

Cultures of procyclic forms of TRUM 397 were established from a stabilate and grown for 14 days. They were added to *Anopheles* cells and forms infective to mice developed after 11 days (Table 2). The culture produced infective forms for 72 days when it was discarded. The prepatent periods in mice infected with this material ranged from 3 to 8 days followed by relapsing and fatal infections. Two of the 17 mice inoculated with trypanosomes grown without cells developed non-fatal parasitaemias.

Metacyclic forms collected from an 80-day-old culture were added to flasks containing *Microtus* cells. Patches of long slender bloodstream forms were observed after 2 days and the cultures soon developed the characteristics of the *T. b. rhodesiense* bloodstream form cultures. As shown in Table 3, the number of infective forms produced by a 14-day-old culture was 6.3×10^5/ml in a total population of 3.9×10^6/ml. Mice injected with culture samples on days 3, 8 and 37 developed relapsing infections similar to those produced by the parent stock. The cultures were terminated after 45 days.

Table 2. Infectivity reacquisition of *T. b. brucei* TRUM 397 and *T. b. rhodesiense* TRUM 601 cultivated at 27°C with *Anopheles gambiae* cells

Stock TRUM	Days with cells before infectivity restoration	Period of infectivity (days)	Infections in mice		
			No infected / No inoculated	Prepatent period (days)	Time to death (days)
397	11[b]	72[c]	17 / 17	3–8	14–28
Control[a]	63	5	2 / 17	5–6	
601	12[b]	63[c]	10 / 10	1–7	13–26
Control[a]	0	0	0 / 5		

[a] Control = trypanosome culture without cells
[b] First inoculated
[c] Cultures terminated

Table 3. Estimation of the number of trypanosomes in and the infectivity (ID_{63}) of samples of bloodstream forms[a] of *T. b. brucei* TRUM 397 and *T. b. rhodesiense* TRUM 589 grown at 37°C with *Microtus montanus* cells

Stock TRUM	Days in culture	No of trypanosomes per ml	No of infective trypanosomes per ml
397	14	3.9×10^6	6.3×10^5
589	21	6.9×10^5	5.4×10^4

[a] Trypanosomes in the supernatant medium

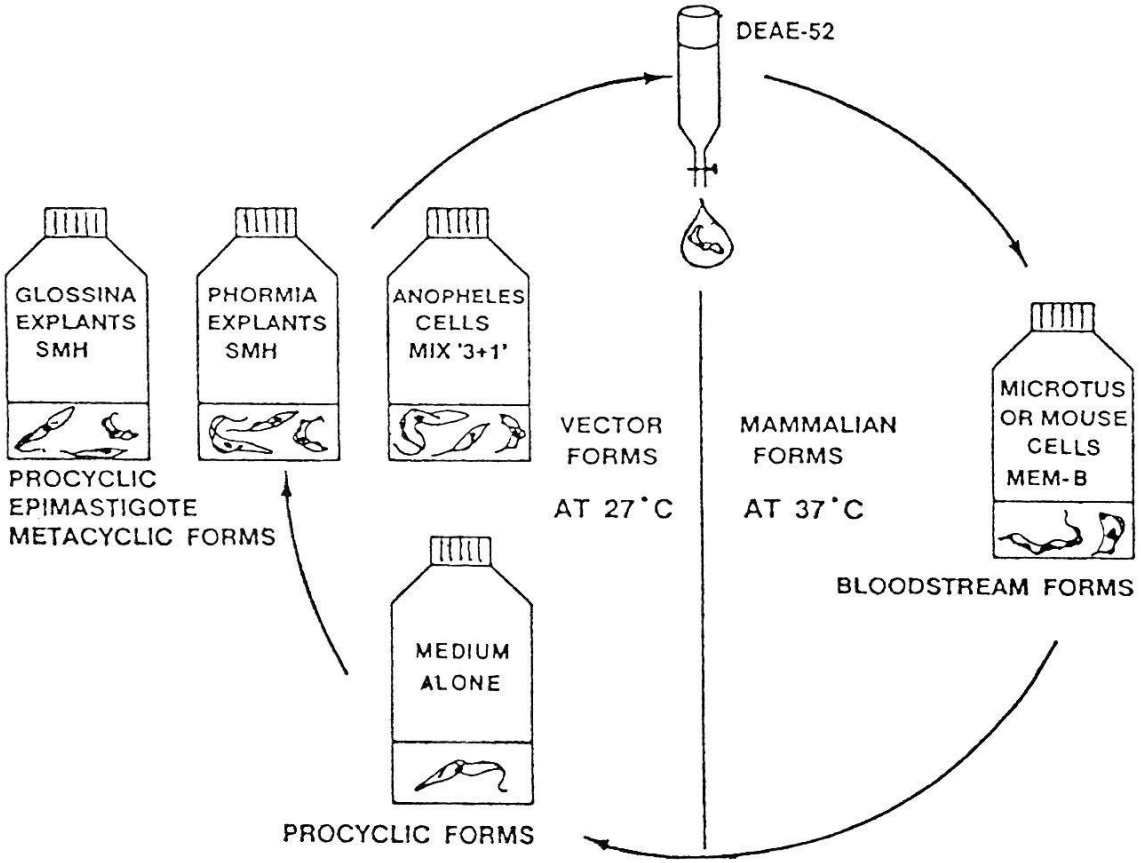


Fig. 6. Schematic diagram of the culture systems.

Bloodstream forms from an 8-day-old culture were transferred to SMH medium and incubated at 27°C. Within 4 days they had transformed into non-infective forms and on the 16th day were added to *Phormia* abdomen tissue cultures. The population became infective after 20 days.

On the 10th day bloodstream forms from an aforementioned culture were passaged into a monolayer of cells from 12-day-old CD-1 mouse embryos and grown at 37°C in medium MEMB. The trypanosomes adapted to the mouse cell environment and grew in the biphasic condition. They remained pleomorphic and infective to mice for 37 days when the cultures were discontinued.

A diagram of the various culture systems used in the in vitro cyclical development is presented in Fig. 6.

Discussion

It is evident from these studies that in vitro-produced bloodstream forms of *T. b. brucei* and *T. b. rhodesiense* could initiate cultures of tsetse fly stages grown with insect tissues or cells. Furthermore, metacyclic forms harvested from these latter cultures transformed into bloodstream forms in mammalian cell feeder layers. These studies extend those of Hirumi et al. (1980) and Hill and Hirumi (1983) in which the culture system remained the same except that transformation from mammalian to tsetse fly forms of *T. b. brucei* was con-

trolled by decrease in incubation temperature. The prolonged period of up to 8 days for complete development of metacyclic into bloodstream forms, and 5 days for bloodstream into procyclic stages observed in the cultures of Hill and Hirumi (1983) might have been a characteristic of the monomorphic highly virulent stock 427. In our studies transformation of metacyclic into bloodstream forms of pleomorphic stocks occurred within 1–2 days, and within only 6–24 h when bloodstream form cultures were established from metacyclics harvested from tsetse flies (Brun et al., 1979, 1981, 1984). In the studies of Cunningham et al. (1981) the prepatent period in mice inoculated with in vitro-produced metacyclic forms of *T. b. brucei* 427 ranged from 5–14 days compared with 3–7 days from inocula of similar material of pleomorphic stocks.

The numbers of bloodstream forms produced in the supernatant medium of the cultures were similar to those estimated in cultures of *T. b. brucei* sspp. prepared from infected mice (Hirumi et al., 1977; Hill et al., 1978; Balber, 1983, 1984; Baltz et al., 1985) or from metacyclics from tsetse flies (Brun et al., 1979, 1981; Nyindo, 1985). Comparable populations were reported in cultures of *T. evansi* (Zweygarth et al., 1983) and *T. vivax* (Brun and Moloo, 1982) prepared from infected blood. The maximum output of bloodstream trypanosomes from cultures of *T. congolense* prepared from (a) explants of infected calf and rabbit skin lesions (Gray et al., 1979; Ross et al., 1985), (b) infected mouse blood (Hirumi and Hirumi, 1984; Ross et al., 1985) or (c) metacyclic trypanosomes (Gray et al., 1985) was about 1×10^6/ml.

The pleomorphism of the bloodstream forms grown in vitro could explain the results of the infectivity titration (Table 2) since the test sample contained short stumpy forms which are unable to grow in mammalian hosts but are adapted for life in the tsetse fly.

A notable feature was the yield of up to 2.5×10^5/ml metacyclic forms of *T. b. rhodesiense* in a population of 3.3×10^6/ml trypanosomes grown with *Anopheles* cells. This is significantly greater than the output of 8.8×10^3/ml in a suspension of 3.2×10^7/ml of the same stock grown with explants of *Glossina morsitans* (Cunningham, 1986) and 7.9×10^3/ml in a population of 3.2×10^7/ml in *Phormia regina* tissue cultures (Cunningham and Kaminsky, 1986). The difference in the culture systems, i.e. the use of cells versus tissue explants and the quality of the medium might have contributed to the greater proportion of metacyclics. The medium used for the *Anopheles* cells + trypanosome cultures consisted of a mixture of *Anopheles* and procyclic form medium (SMH) and thus reduced the level of components, such as proline, which is a major energy source of procyclic forms. The yields of metacyclic trypanosomes from our cultures were similar to those reported in cultures of *T. congolense* (Gray et al., 1981, 1984) initiated from trypanosomes in the proboscis of tsetse flies and of *T. vivax* (Hirumi et al., 1984) prepared from infected tsetse flies or bovine blood.

Improved and reproducible methods for the cultivation of pathogenic African trypanosomes would provide material for studies on the morphological and antigenic changes associated with loss and reacquisition of infectivity as it occurs in the vector and mammalian hosts. Production of infective trypanosomes in vitro could aid investigations of methods for immunological control of trypanosomiasis. However, organisms can change their characteristics during prolonged cultivation and it is essential that their natural properties are retained in the culture systems.

Acknowledgments

This research was supported by a grant No 5 ROI A120390–02 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service. It is a pleasure to thank Professor B. M. Honigberg for his encouragement during these studies, Dr. A. M. Jordan, Tsetse Research Laboratory, Langford, Bristol, England, for supplies of tsetse flies, and Dr. Imogene Schneider, WRAIR, Washington D.C., for supplies of insect cell lines. Dr. R. Kaminsky is a Feodor Lynen Research Fellow of the Alexander von Humboldt Foundation, West Germany.

Balber A. E.: Primary murine bone marrow cultures support continuous growth of infectious human trypanosomes. Science *220*, 421–423 (1983).

Balber A. E.: Growth of *Trypanosoma brucei gambiense, T. b. rhodesiense* and *T. congolense* in bone marrow cultures of genetically anemic sl/sl^{d} mice. J. Parasit. *70*, 179–180 (1984).

Baltz T., Baltz D., Giroud C. H., Crockett J.: Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense*. The EMBO Journal *4*, 1273–1277 (1985).

Brun R., Moloo S. K.: In vitro cultivation of animal-infective forms of a West African *Trypanosoma vivax* stock. Acta trop. (Basel) *39*, 135–141 (1982).

Brun R., Jenni L., Tanner M., Schönenberger M., Schell K.-F.: Cultivation of vertebrate infective forms derived from metacyclic forms of pleomorphic *Trypanosoma brucei* stocks. Acta trop. (Basel) *36*, 387–390 (1979).

Brun R., Jenni L., Schönenberger M., Schell K.-F.: In vitro cultivation of bloodstream forms of *Trypanosoma brucei, T. rhodesiense* and *T. gambiense*. J. Protozool. *28*, 470–479 (1981).

Brun R., Hecker H., Jenni L., Moloo S. K.: A quantitative ultrastructural study on the transformation of *Trypanosoma brucei* metacyclic to bloodstream forms in vitro. Acta trop. (Basel) *41*, 117–129 (1984).

Cunningham I.: New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. J. Protozool. *24*, 325–329 (1977).

Cunningham I.: Infectivity of *Trypanosoma rhodesiense* cultivated at 28° C with various tsetse fly tissues. J. Protozool. *33*, 226–231 (1986).

Cunningham I., Honigberg B. M.: Infectivity reacquisition by *Trypanosoma brucei brucei* cultivated with tsetse salivary glands. Science *197*, 1279–1282 (1977).

Cunningham I., Kaminsky R.: Development of metacyclic forms of *Trypanosoma brucei* sspp. in cultures containing explants of *Phormia regina* Meigen. J. Parasit. *72*, 944–948 (1986).

Cunningham I., Honigberg B. M., Taylor A. M.: Infectivity of monomorphic and pleomorphic *Trypanosoma brucei* stocks cultivated at 28° C with various tsetse fly tissues. J. Parasit. *67*, 391–397 (1981).

Dethier V. G., Goldrich N.: Blowflies: alteration of adult taste responses by chemicals present during development. Science *173*, 242–244 (1971).

Gardiner P. R., Lamont L. C., Jones T. W., Cunningham I.: The separation and structure of infective trypanosomes from cultures of *Trypanosoma brucei* grown in association with tsetse fly salivary glands. J. Protozool. *27,* 182–185 (1980).

Gray M. A., Brown C. D. G., Luckins A. G., Gray A. R.: Maintenance of infectivity of *Trypanosoma congolense* in vitro with explants of infected skin at 37°C. Trans. roy. Soc. trop. Med. Hyg. *73,* 406–408 (1979).

Gray M. A., Cunningham I., Gardiner P. R., Taylor A. M., Luckins A. G.: Cultivation of infective forms of *Trypanosoma congolense* from trypanosomes in the proboscis of *Glossina morsitans.* Parasitology *82,* 81–95 (1981).

Gray M. A., Ross C. A., Taylor A. M., Luckins A. G.: In vitro cultivation of *Trypanosoma congolense:* the production of infective metacyclic trypanosomes in cultures initiated from cloned stocks. Acta trop. (Basel) *41,* 343–354 (1984).

Gray M. A., Ross C. A., Taylor A. M., Tetley L., Luckins A. G.: In vitro cultivation of *Trypanosoma congolense:* the production of infective forms from metacyclic trypanosomes cultured on bovine endothelial cell monolayers. Acta trop. (Basel) *42,* 99–111 (1985).

Hill G. C., Hirumi H.: African trypanosomes. In: In vitro cultivation of protozoan parasites, ed. by J. B. Jensen, p. 193–219. CRC Press Inc., Florida, USA 1983.

Hill G. C., Shimer S. P., Caughey B., Sauer S.: Growth of infective forms of *T. rhodesiense* in vitro, the causative agent of African trypanosomiasis. Science *202,* 763–765 (1978).

Hirumi H., Hirumi K.: Continuous cultivation of animal-infective bloodstream forms of an East African *Trypanosoma congolense* stock. Ann. trop. Med. Parasit. *78,* 327–330 (1984).

Hirumi H., Doyle J. J., Hirumi K.: Cultivation of bloodstream *Trypanosoma brucei.* Bull. WHO *55,* 405–409 (1977).

Hirumi H., Hirumi K., Nelson R. T., Bwayo J. J.: Present status of in vitro cultivation of animal-infective African trypanosomes. In: The in vitro cultivation of the pathogens of tropical diseases. Tropical Diseases Research Series No 3, p. 165–200. Schwabe, Basel, 1980.

Hirumi H., Hirumi K., Nelson R. T., Moloo S. K.: Propagation of metacyclic-like forms of East African *Trypanosoma vivax.* J. Protozool. *31,* 7A (Abstr. No 24) (1984).

Jones T. W., Cunningham I., Taylor A. M., Gray A. R.: The use of culture-derived metacyclic trypanosomes in studies on the serological relationships of stocks of *Trypanosoma brucei gambiense.* Trans. roy. Soc. trop. Med. Hyg. *75,* 560–565 (1981).

Lanham S. M., Godfrey D. G.: Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Parasitology *28,* 531–534 (1970).

Lumsden W. H. R., Cunningham M. P., Webber W. A. F., van Hoeve K., Walker P. J.: A method for the measurement of the infectivity of trypanosome suspensions. Exp. Parasit. *14,* 269–279 (1963).

Novy F. G., MacNeal W. J.: On the cultivation of *Trypanosoma brucei.* J. infect. Dis. *1,* 1–30 (1904).

Nyindo M.: Specific immunity in rats inoculated with *Trypanosoma brucei brucei* derived from tsetse salivary glands and cultured in vitro. Acta trop. (Basel) *42,* 123–126 (1985).

Pittam M. D.: Medium for in vitro culture of *T. rhodesiense* and *T. brucei,* Appendix, p. 127–128. In: Dixon H., Williamson J.: The lipid composition of blood and culture forms of *Trypanosoma lewisi* and *Trypanosoma rhodesiense* compared with that of their environment. Comp. Biochem. Physiol. *33,* 111–128 (1970).

Reid H. V., Burridge M. J., Pullan N. B., Southerst R. W., Wain E. B.: Survey of trypanosome infections in domestic and wild animals in areas of East Africa. Brit. Vet. J. *126,* 622–626 (1970).

Ross C. A., Gray M. A., Taylor A. M., Luckins A. G.: In vitro cultivation of *Trypanosoma congolense:* establishment of infective mammalian forms in continuous culture after isolation from the blood of infected mice. Acta trop. (Basel) *42,* 113–121 (1985).

Tobie E. J.: Cultivation of mammalian trypanosomes. J. Protozool. *11,* 418–423 (1964).

Varma M. G. R., Pudney M.: The growth and serial passage of cell lines from *Aedes aegypti* (L) larvae in different media. J. med. Entomol. *6,* 432–439 (1969).

Zweygarth E., Ahmed J. S., Rehbein G.: Cultivation of infective forms of *Trypanosoma brucei evansi* in a continuous culture system. Z. Parasitenk. *69,* 131–134 (1983).