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In Vitro Cultivation of Blood Stream Trypomastigotes of *Trypanosoma vivax* without Feeder Cell Layers

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ABSTRACT. Bloodstream trypomastigotes (BSFs) of 2 clones (IL 1392 and IL 3671) of *Trypanosoma vivax* were cultured without feeder layers in 3 systems. System I: metacyclic-producing cultures of *T. vivax* IL 1392 were initiated with BSFs derived from infected mice at 27 ^{O}C in the presence of feeder layers using TVM-1 medium. Metacyclics harvested were then transferred to flasks containing feeder cells and maintained at 34 ^{O}C using TVM-22 medium. Under such conditions, the metacyclics transformed to BSFs. Bloodstream trypomastigotes which were then transferred to new flasks, continued to grow in HMI-162 medium without feeder cells. The maximum density of the BSFs and their shortest population doubling time were 3.5 x 10^6 / ml and 13.5 h, respectively. System II: metacyclic-producing cultures of *T. vivax* IL 3671 were initiated with BSFs derived from infected cattle, without feeder layers at / 27 ^{O}C using HMI-107 medium. Metacyclics were then transferred to the axenic culture conditions established in System I. They also transformed to BSFs and multiplied in HMI-162 and HMI-163 media. System III: cultures were initiated with proboscides of *Glossina morsitans centralis* which were infected with *T. vivax* IL 3671, without feeder layers at 34 ^{O}C using HMI-162 medium. Metacyclics emerged from the proboscides, transformed to BSFs and continued to grow in HMI-163 medium. The maximum density and the population doubling time of IL 3671 BSFs in HMI-163 medium were 1.8 x 10^6 / ml and 16.1 h, respectively.

INTRODUCTION

African trypanosomiases are caused by protozoan blood parasites, major species being *Trypanosoma brucei*, *T. congolense* and *T. vivax* which are mainly transmitted by tsetse fly vectors (*Glossina* spp). Basic procedures that support the growth of these trypanosomes in vitro have been well developed during the past 15 years since the first continuous culture of bloodstream trypomastigotes (BSFs) of *T. brucei* was achieved by Hirumi et al. (1977). All the stages of the 3 parasites, at least those of stocks and/or clones tested to date, can be cultured in vitro (Hirumi et al. 1980; Brun and Jenni 1987; Gray et al. 1987). Using these procedures, BSFs of the three species can be propagated only in the presence of mammalian feeder cell layers. However, in vitro systems that support the growth of the BSFs in the absence of feeder cells have recently become indispensable for studying factors that regulate stage-specific transformations, as well as for investigating the sensitivity of the parasites to trypanocidal drugs and the study of the mechanisms involved in drug resistance. In vitro systems which support axenic cultivation of *T. brucei* BSFs were reported by Baltz et al. (1985) and Duszenko et al. (1985). These systems were further improved by introducing HMI-9 medium (Hirumi and Hirumi 1989) which was modified from Iscove's modified Dulbecco's minimum essential medium (Iscove's DMEM) by supplementing with bathocuproine sulfonate, L-cysteine, hypoxanthine, 2-mercaptoethanol, sodium pyruvate, thymidine, and 10% (v/v) fetal bovine serum and 10% (v/v) Serum Plus$^{(TM)}$ (Hazleton Biologics, Lenexa, KS, USA). Yabu et al. (1989) have also reported axenic cultivation of a *T. b. gambiense* stock by using a modified

DMEM, containing bathocuproine sulfonate and L-cysteine. Furthermore, an in vitro system for cultivating *T. congolense* BSFs without feeder cells was established by using HMI-93 medium which was modified from HMI-9 medium (Hirumi and Hirumi 1991). More recently, an in vitro system for the axenic cultivation of BSFs of 4 *T. vivax* stocks (IL 1392, IL 32683 IL 3269 and KETRI 2501) was reported by Zweygarth et al. (1991). In this system, BSFs were firstly adapted to in vitro conditions in the presence of feeder cell layers and were then maintained in axenic culture condition up to 60 days in 24-well culture plates using 2 modifications of MEM which contained supplements similar to those that were used previously in the axenic cultivation of *T. brucei* and/or *T. congolense.* However, the maximum density of BSFs obtained in the systems was low (5×10^5 / ml) and the shortest population doubling times of the 4 stocks were rather long (21, 18, 29 and >48h, respectively).

In the present study, 3 axenic culture systems (Systems I, II, and III) that support continuous growth of BSFs of 2 *T. vivax* clones (IL 1392 and IL 3671) in $25cm^2$ T-type (T-25) flasks were established by using HMM62 and HMI-163 media which were further modified from HMI-93 medium. In System I, BSFs transformed from in vitro-produced metacyclics were maintained in the presence of feeder layers prior to the axenic cultivation, while, in Systems II and III, trypanosomes were cultivated without use of feeder layers at any stage. The maximum densities of the BSFs achieved in these systems were higher and the population doubling times were shorter than those reported by Zweygarth and coworkers (1991).

MATERIALS AND METHODS

Trypanosomes. A West African clone IL 1392 of *T. vivax* is a derivative of a stock Zaria Y486, isolated from a Zebu cow, (*Bos indicus*), in Zaria, Nigeria in 1973 and is infective for laboratory rodents (Leeflang et al. 1976). An East African clone IL 3671 of *T. vivax* is a derivative of the primary isolate TVS, obtained from a naturally infected Friesian cow (*B. taurus*) at a dairy farm in Bamburi, Mombasa District, Coast Province, Kenya in 1986 (Schonefeld et al. 1987). It is not infective for laboratory rodents, but produces an haemorragic disease in cattle.

Mice. Inbred Balb/c mice, aged between 5 and 6 months, were reared at ILRAD and were used to prepare *T. vivax* IL 1392-infected blood samples for initiating primary cultures in System I, as well as to test the infectivity of in vitro-propagated BSFs of this stock.

Cattle. Three Boran (*B. indicus*) and 3 Ayrshire (*B. taurus*) cattle, aged between 6 and 10 months, born at ILRAD's Farm in a tsetse fly-free area of Kenya, were reared at ILRAD. An Ayrshire steer was inoculated (I.V,) with *T. vivax* IL 3671 and was used to prepare infected blood samples for initiating primary cultures in System IL Two other Ayrshire cattle were used to test the infectivity of IL 3671 BSFs propagated in Systems II and III. A Boran steer was also inoculated (I.V.) with *T. vivax* IL 3671 and was used to infect tsetse flies (*Glossina morsitans centralis*) by feeding as described below. Two other Boran cattle were donors of bovine plasma that was used to supplement a culture medium (Table 1) as well as to separate in vitro-produced metacyclics from epimastigotes in Systems I and II as described below.

Goats. Most young goats used were between 3 and 10 months of age and were East African Massai and Galla crossbred, born and reared at ILRAD. Some other crossbred animals, aged about 6 months, were brought from the Mount Kenya region, which is known to be free from tsetse flies and trypanosomiases, and were maintained at ILRAD. One of the goats born at ILRAD was used to test the infectivity of IL 1392 BSFs propagated in System L All the others were used as blood donors for serum preparation.

Tsetse flies. *Glossina m. centralis* were bred and reared at ILRAD (Moloo et al. 1985). Teneral (young, unfed) flies were fed on the cattle infected with *T. vivax* IL 3671 during the first peak of parasitaemia and were maintained by feeding on the same animal every day except at week ends. Twenty five days following the infective bloodmeal, the surviving flies were allowed to salivate singly to warm slides at 37 ^{O}C to identify those with mature infections. Tsetse flies with metacyclics in their salivary probes were used to initiate cultures in System III.

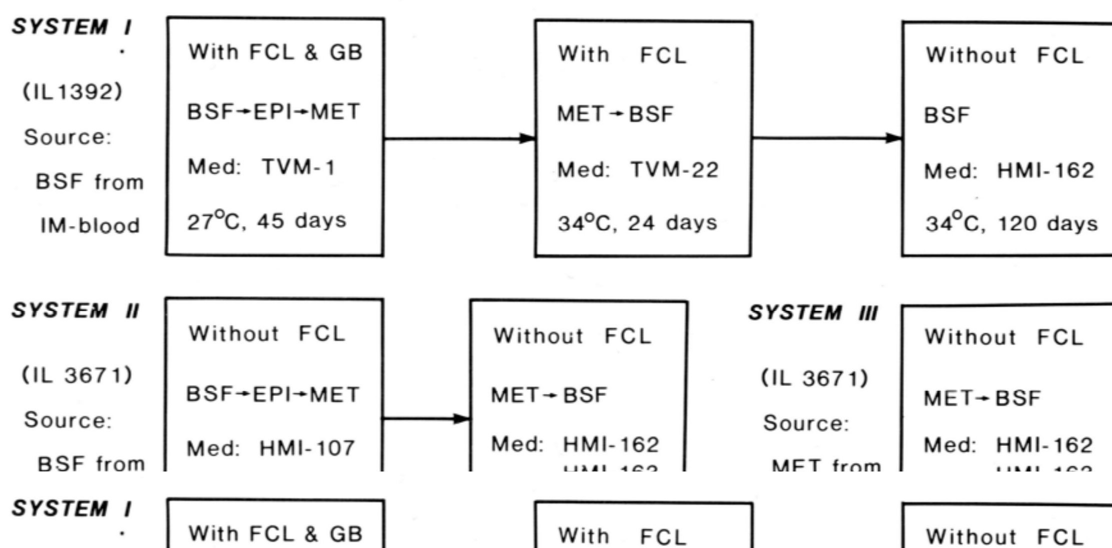


Fig. 1. Summary of Systems I, II and III used for cultivating bloodstream trypomastigotes (BSF) of West African (IL 1392) and East African (IL 3671) clones of Trypanosoma vivax without feeder cell layers (FCL), EPI: epimastigotes, GB: Matrex Gel Green-A beads (Amicon); MET: metacyclics; Med: medium listed in Table 1; IB-blood: infected bovine blood; IM-blood: infected mouse blood; IT-prob.: infected tsetse proboscides,

Culture media. Details of culture media, TVM-1, TVM-22, HMI-93, HMI-107, HMI-162 and HMI-163 are listed in Table 1. The TVM media were based on Eagle's minimum essential medium (MEM) and were used to cultivate the trypanosomes in the presence of feeder cell layers as described below: TVM- 1 medium for metacyclic-producing epimastigote populations of *T. vivax* IL 1392, and TVM-22 medium for IL 1392 BSFs. The HMI media were based on Iscove's DMEM, modified from HMI-93 that was originally established for the axenic cultivation of *T. congolense* BSFs (Hirumi and Hirumi 1991), and were used without feeder cell layers: HMI-107 medium for metacyclic-producing epimastigote populations of *T. vivax* IL 3671, HMI-162 medium for BSFs of IL 1392 and IL 3671, and HMI-163 medium for BSFs of IL 3671.

Feeder cell layers. *Microtus montanus* embryonic fibroblast-like cells were prepared in T-25 flasks ($25cm^2$ T-type Tissue Culture Flasks; Falcon, Cat. No. 3013, Lincon Park, NJ, USA) as described by Brun et al. (1981). When the cells formed confluent layers (3-5 days old subculture), they were used for trypanosome cultivation.

Preparation of Green-A beads. Matrex Gel Green-A beads$^{(TM)}$ (crosslinked 5% agarose with covalently coupled dye; Amicon, Danvers, MA, USA) were pelleted by centrifuging 10 ml of the original suspension at 1000g for 5 min, resuspended in 5 ml of 70% ethanol in water and were allowed to settle. After repeating the ethanol washing 3 times, the beads were resuspended in 10 ml of 70% ethanol and were kept overnight at 4 ^{O}C The beads were then washed twice with MEM, resuspended in 5 ml of MEM containing Penicillin (100 U/ml)-Streptomycin (100 µg/ml) mixture (GIBCO Cat. No.600-5145AE) and were stored at 4 ^{O}C until use. When primary cultures in System I were prepared, the beads were resuspended in 5 ml of fresh TVM-1 medium, without antibiotics, and 0.5 ml of the suspension was added to each culture.

Isolation of BSFs from infected blood. In System I, IL 1392-infected mouse blood, containing 70-80 BSFs per 400x microscope field (as examined on a thin wet film of tail blood), was collected by cardiac puncture at a rising parasitaemia (usually a day before the peak of parasitaemia) using anticoagulant (heparin, final concentration of 5-10 I.U./ml; Novo Industri, Bagsvaerd, Denmark). The infected blood was mixed with TVM-1 medium (1 part blood and 9 parts medium) in 5 ml Micro Product Vials

Table 1. Media for cultivating *Trypanosoma vivax*

Medium code number	Feeder cell layers[a]	Forms of parasites[b]	Basal medium[c]	Supplements[d]												
				Serum (%, v/v)				Other (mM)								
				FBS	YGS	BP	SP	BAC	CYS	GLU	HYP	2ME	PRO	PYR	THY	NEA (100x)
TVM-1	+	EPI & MCF	MEM	20						2						
TVM-22	+	BSF	MEM		8	12				2	0.2					1 (%)
HMI-93[e]	-	(*T. congolense*)	IMDM		20		5	0.05	1.50		0.5	0.12		1	0.16	·
HMI-107	-	EPI & MCF	IMDM	20				0.03	0.30		0.2	0.14	0.6	1		
HMI-162	-	BSF	IMDM		20			0.01	0.15		0.2	0.07		1	0.04	
HMI-163	-	BSF	IMDM		20		3	0.03	0.80		0.2	0.07		1	0.10	

a Presence (+) or absence (-) of feeder cells, embryonic fibroblast-like cells of *Microtus montanus*.

b EPI, epimastigotes; MCF, metacyclic forms; BSF, bloodstream trypomastigotes.

c MEM, Eagle's minimum essential medium (GIBCO, Paisley, Scotland, UK); IMDM, Iscove's modified Dulbecco's MEM (Flow Laboratories, Irvine, Scotland, UK).

d FBS, fetal bovine serum (Northumbria Biologicals, Lot No. S102, Cramlington, U.K.); YGS, young goat serum (prepared at ILRAD); BP, bovine plasma (prepared at ILRAD); SP, Serum Plus(TM) (Hazleton Biologics, Lenexa, KS, USA); BAC, bathocuproinedisulfonic acid (Sigma, St Louis, MO, USA); CYS, L-cysteine (Sigma); GLU, L-glutamine (GIBCO); HYP, hypoxanthine (Calbiochem, La Jolla, CA, US); 2ME, 2-mercaptoethanol (BDH Chemicals, Poole, England, UK); PRO, L-proline (E. Merck, Darmstadt, Germany); PYR, sodium pyruvate (Sigma); THY, thymidine (Sigma); NEA, Eagle's non-essential amino acids (100x) (1%, v/v) (GIBCO).

e Originally designed for the axenic cultivation of *T. congolense* bloodstream trypomastigotes (Hirumi and Hirumi 1991).

(Wheaton Cat. No. 986259, Millville, NJ, USA) and was centrifuged at 120g for 7 min at 4°C. Supernatants were collected and pellets were resuspended in fresh medium. This procedure was repeated 3 times. The supernatants containing most BSFs and a small number of red blood cells were pooled and were used to initiate primary cultures of IL 1392 metacyclic-producing epimastigote populations. In System II, IL 3671infected bovine blood, containing 10-20 BSFs per 400x microscope field, was collected from the jugular vein at the peak of parasitaemia using 50 ml syringes containing heparin (final concentration of 10 I.U./ml). The infected blood was mixed with HMI-107 medium (1 part blood and 9 parts medium) in 10 ml conical centrifuge tubes (Sterilin Cat. No. 144AS, Hounslow, UK) and was centrifuged at 150g for 15 min. The supernatants were collected and pooled after 3 centrifugation steps as described above, and were used to initiate primary cultures of IL 3671 metacyclic-producing epimastigote populations.

Isolation of in vitro-produced metacyclics. Metacyclics produced in Systems I and II were isolated from epimastigotes by the "bovine plasma aggregation method" described in outline earlier (Hirumi et al. 1985). Culture fluids were pooled from metacyclic-producing cultures. In detail, after adding bovine plasma (10% v/v), the culture fluids containing epimastigotes and metacyclics were incubated at 27 ^{O}C for 60 min. During the incubation period, epimastigotes were aggregated forming clumps, while metacyclics remained swimming freely. The metacyclics were then separated from the epimastigote clumps by passing the trypanosome suspension at 1g through a filter of 5µm pore size, 9 cm in diameter (Millipore Cat. No. SMWP 090 25, Bedford, MS, USA) which was held in a RF-20 Radial Flow Cell (Nucleopore Cat. No. 552014, Pleasant, CA, USA). The culture fluid (up to 80 ml pooled from up to 20 T-25 flasks) was applied to 1 filter and tile metacyclics were eluted with phosphate-buffered saline solution (pH 8.1) containing 1 % (w/v) glucose (PSG) (Lanham and Godfrey 1970) until no metacyclics were observed in the eluting PSG drop. The metacyclics were then collected by centrifugation at 750g for 15 min in 10 ml conical centrifuge tubes and were resuspended in a culture medium and adjusted to a desirable density (1-6 x 10^{6}/ml).

Examination of cultures. The morphological appearance of trypanosomes, as well as their growth patterns in culture, were examined at various stages of cultivation using an inverted phase-contrast microscope (Nikon DIAPHOTO-TMD, Nippon Kogaku, Tokyo, Japan) and phase-contrast micrographs were taken using a Nikon MICROPHOT UFX-11 equipped with Nikon SPEEDLIGHT SBM (Nippon Kogaku). Morphological characteristics of trypanosomes were also examined by preparing Giemsa-stained smears of trypanosomes in culture fluids, as well as of those isolated by the bovine plasma aggregation method. The smears were quickly air dried, fixed with 5% (v/v) formalin in PSG for 20 min, post-fixed with methanol for 20 min, stained with 5% Giemsa solution (E. Merck Cat. No. 9204, Darmstadt, Germany) in distilled water for 20 min, washed in distilled water and air dried. The fixation with 5% formalin facilitated the distinction of the kinetoplast from large cytoplasmic granules which often appeared in insect forms. The stained smears were examined using a Nikon MICROPHOT-FX light microscope (Nippon Kogaku). Bloodstream forms of *T. vivax* IL 1392 obtained on day 61 in the axenic culture were fixed for 2 h in an equal volume of fixative containing 2.5% (v/v) glutaraldehyde, 2% (v/v) formaldehyde and 0.03% (w/v) picric acid in 0.1 M phosphate buffer (pH 7.4). The samples were pelleted and post-fixed in 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 h, washed in 0.05 M maleate buffer (pH 5.2) and treated with 1% (w/v) uranyl acetate in maleate buffer for 1 h. After a brief wash in distilled water, the samples were dehydrated with ethanol and embedded in Epon-Araldite. Thin sections were stained with uranyl acetate followed by lead citrate and examined using a Zeiss EM10A electron microscope (Oberkochen, Germany). Numbers of trypanosomes were determined by haemocytometer and their population doubling times were calculated using the equation of Hayflick (1973).

Experimental approaches. Three axenic culture systems established in the present study for cultivating *T. vivax* BSFs are summarized in Fig. 1. Other details of specific procedures are described on appropriate

pages in the Results section.

RESULTS

In vitro cultivation

System I was established in 3 steps: (1) production of metacyclics of *T. vivax* IL1392 in the presence of feeder cell layers at 27 ^{O}C, (2) transformation of the metacyclics to BSFs and propagation of the BSFs in the presence of feeder cells at 34 ^{O}C and (3) continuous cultivation of the in vitro-transformed BSFs in the absence of feeder cells at 34 ^{O}C (Fig. 1).

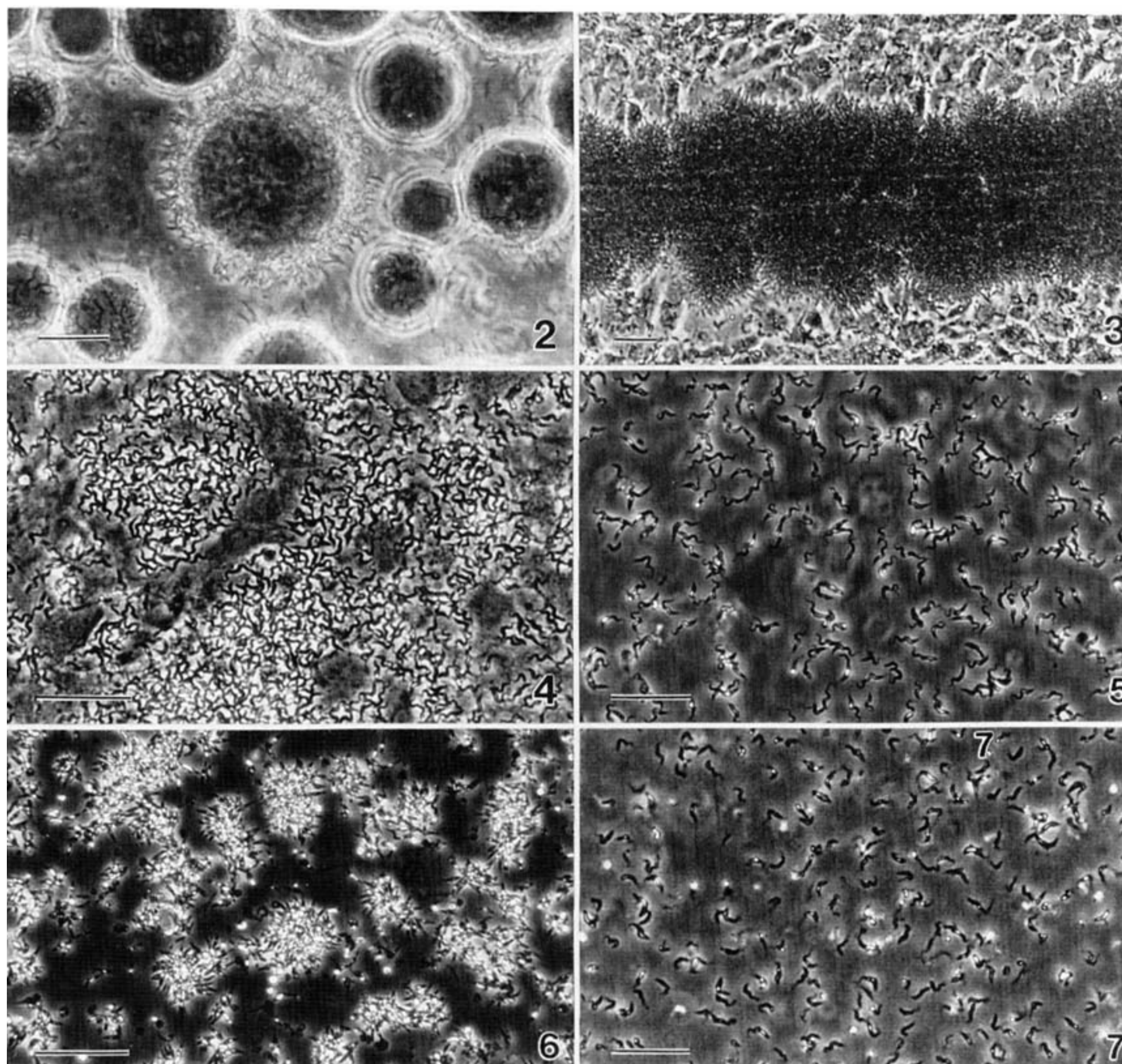
Step 1. A number of culture conditions, including culture media, types of feeder layer cells, attachment factors, microcarrier beads, culture vessels, CO_2 concentrations, temperatures and different-life cycle stages of the parasites, have been previously tested for cultivating metacyclic-producing epimastigote populations of *T. vivax* using 2 West African (IL 1392 and IL 317L) and 4 East African (IL 2133, IL 2241, IL 2292 and IL 2337) stocks derived from infected blood of respective hosts and/or infected tsetse proboscides (Hirumi et al. 1985, 1988). The optimum culture conditions standardized in these studies were used in the present study.

A total of 6 primary cultures were initiated in T-25 flasks (Costar Cat. No.3055, Cambridge, MA, USA) which contained confluent cell layers of M. montanus embryonic fibroblast-like cells and Matrex Gel Green-A beads in TVM-1 medium by placing BSFs (3xl0^{7}/flask) which were isolated from infected mouse blood and suspended in TVM-1 medium. Fresh TVM-1 medium was added to make a total volume of 5 ml per flask and the cultures were incubated at 27 ^{O}C without change of the medium for 3 days. The BSFs transformed to elongated trypomastigotes and attached to the surface of the beads (Fig. 2) by their anterior end during the initial period. These elongated forms are known to lack variable surface glycoprotein and were not infective for rodents in our earlier studies (Hirumi el al. 1985, 1988). The elongated trypomastigotes further transformed to epimastigotes which were at first elongated and subsequently became short during the next 5-7 days when 1-2 ml of medium/flask was changed every other day. By days 10-12 onwards, many short epimastigotes detached from the beads and then reattached to the surface of feeder cells as well as to open spaces of plastic amongst the feeder layers which were made by scraping with a tip of Pasteur pipette from day 10 onwards. The short epimastigotes continued to proliferate forming clusters (Fig. 3) for 2-3 months when the medium was changed 3 times a week.

From day 20 onwards, subcultures of the epimastigote populations were made by transferring epimastigote-containing culture fluids to new flasks (1-2 ml/flask) in which the feeder layers had been partially scraped off. The flasks then received fresh medium up to 5 ml/flask and were maintained in the same manner as for the original flasks. More than 250 subcultures were made during the course of the study. The morphological appearance of epimastigote clusters as observed by phase-contrast microscopy was similar to that of the clusters grown on the internal surface of infected proboscides of tsetse flies.

From day 12 onwards, short trypomastigotes which were morphologically similar to metacyclics from the saliva of infected tsetse flies, and which were infective for rodents in the earlier studies (Hirumi et al. 1985 and 1988), appeared among the epimastigotes and increased in number as the duration of culture was prolonged. They were collected 1-2 times a week from day 20 onwards by the bovine plasma aggregation method. No dividing forms were seen among the short trypomastigotes. These trypomastigotes were thus referred to as metacyclics.

Step 2. Using metacyclics of *T. vivax* IL 1392 obtained in Step 1, a total of21 culture media modified from TVM-1 medium were tested for their ability to support (1) the transformation of the metacyclics to BSFs, and (2) the continuous proliferation of the transformed BSFs in the presence of feeder cell layers at 34 ^{O}C. Among these, TVM-22 medium (Table 1) was established to be the best medium for the transformation as well as for supporting continuous growth (data not shown). The metacyclics suspended in TVM-22 medium (10^{6}/ml) were then placed in T-25 flasks containing con fluent feeder layers (3 ml/flask) and were incubated at 34°C. The metacyclics rapidly attached to the surface of feeder cells by their anterior end, transformed to BSFs within 18 h and continued to proliferate. Most of the BSFs attached to the surface of feeder cells (Fig. 4) while some detached and formed small clumps. The cultures



Figs. 2-7. Various stages of *Trypanosoma vivax* in Systems I, II, and III. Bars indicate 50μm. Fig.2: Elongated trypomastigotes of IL 1392 attaching to Matrex Gel Green-A beads. Culture was maintained in TVM-1 medium for 3 days at 27 ^{O}C in the presence of feeder layers in System I, Step 1. Fig.3: Large cluster of short epimastigotes of IL 1392 maintained for 32 days in System I as mentioned above. Fig.4: Bloodstream trypomastigotes of IL 1392 maintained in TVM-22 medium for 33 days at 34 ^{O}C in the presence of feeder layers in System I, Step 2. Fig.5: Bloodstream trypomastigotes of IL 1392 maintained in HMI-162 medium at 34 ^{O}C for 92 days without feeder layers in System I, Step 3. Most bloodstream trypomastigotes attached to (he bottom of flask. Fig.6: Small clusters of short epimastigotes of IL 3671 in a 7-day-old subculture. Culture was maintained in HM1-1G7 medium for 40 days at 27 ^{O}C without feeder layers in System H, Step I, Fig,7: Bloodstream trypomastigotes of IL 3671 maintained in HMI-163 medium at 34 ^{O}C without feeder layers, 70-day-otd culture in System III. Most bloodstream trypomastigotes of IL 3671 were swimming freely in the medium.

were maintained by replacing culture fluids (2-2.5 ml/flask) with the same volume of fresh medium every 24 h. When the population density became high (>4x 10^6 BSFs/ml), many BSFs detached from the feeder layers and formed clumps which subsequently degenerated. Thus, the maximum population density attained was kept lower than $4x10^6$/ml by reducing the number of attached BSFs at the time of medium change. The subcultivation was carried out by the following procedures: (i) discard of the culture fluids containing detached clumps, (ii) replacement with fresh medium and resuspension of attached BSFs by gentle shaking, (iii) adjustment of the number of BSFs to $5x10^5$/ml in the fresh medium, (iv) transfer to new T-25 flasks (3ml/flask) containing feeder cell layers, and (v) incubation at 34 ^{O}C. More than 200 subcultures

were made during the course of te study.

Step 3. Using *T. vivax* IL 1392 BSFs obtained in Step 2, a total of 69 culture media modified from HMI-93 medium were tested, using Plastek M T-25 flasks (MatTek, Ashland, MA, USA) in a number of experiments, for their ability to support the continuous growth of the BSFs in the absence of feeder cell layers at 34 ^{O}C. Although most media supported a poor, or no, growth of the BSFs, HMI-162 medium (Table 1) was found to be the best medium for supporting growth (data not shown). Thus, the axenic cultivation of IL 1392 BSFs was standardized using HMI-162 medium, (HMI-163 medium, described below, was a later modification).

In the standardized method, HMI-93 medium in cultures prepared in Step 2 was replaced with fresh HMI-162 medium (2.5 ml/flask) and then attached BSFs were resuspended by gentle shaking. The BSF suspensions (usually containing 1-2x10^6 BSFs/ml) were transferred to new Plastek M T-25 flasks (from 1 original flask to 1-2 new flasks) and were then incubated in the absence of feeder cell layers at 34 ^{O}C for 24 h. During this period, most BSFs attached to the bottom of the flasks (Fig.5) while a small number of BSFs were swimming freely in the medium. Occasionally, a few feeder cells were also transferred together with BSFs from the original flasks and attached to the new flasks. In order to eliminate possible contamination of the feeder cells in the axenic cultures, the culture fluids in the flasks were replaced with fresh HMI-162 medium 24 h after the initiation and attached BSFs were resuspended again by gentle shaking and were transferred to new flasks. By repeating this procedure 3 times during the initial 3 days, possible contaminations with feeder cells were completely eliminated. The BSF populations were thereafter maintained by replacing culture medium (2.5 ml/flask) every 24 h until the experiment was terminated on day 120 of the axenic cultivation.

When the population density reached over 3.5x10^6 BSFs/ml, many BSFs detached from the bottom and formed small clumps, indicating the occurrence of overpopulation in such a culture. An increase of volume of the culture medium, up to 5 ml per flask, did not improve the maximum population density, suggesting that an optimum amount of dissolved oxygen for the continuous growth of attached BSFs around the bottom might be reduced when the depth of culture medium was increased. Thus, each culture was maintained with 2.5 ml of the medium and the population densities were kept lower than 3.5x10^6/ml either by reducing the number of attached BSFs by gentle shaking when the medium was changed or by subcultivating BSFs to new flasks.

During the course of this study, a total of 62 subcultures were made by the following procedures: (i) resuspension of attached BSFs in fresh medium, (ii) adjustment of the number of BSFs to a desirable seeding density (usually 10^6/ml), (iii) transfer to new flasks (2.5 ml/flask), (iv) keeping caps of the flasks loose so that the medium was exposed to 2% CO_2 in air in a CO_2 incubator (Heraeus Model No. B5060EK/CO_2, Hanau, F.R. Germany) at 34 ^{O}C for 1-2 h (until the colour of the phenol red in the medium indicates approximately pH 7.4) immediately after subcultivation and then close the caps tightly, and (v) incubation at 34°C.

Growth characteristics of IL1392 BSFs in the axenic culture were examined in 2 groups of 4 subcultures seeded with 10^6 BSFs/ml (2.5 ml/flask) on days 69 and 89 of the axenic cultivation, respectively. Average population densities in the 2 groups 24 h after subcultivation were $3.43 \pm 0.22 \times 10^6$ BSFs/ml and $3.38 \pm 0.22 \times 10^6$ BSFs/ml, and their population doubling times were 13.5 h and 13.7 h, respectively.

System II was established in 2 steps: (1) production of metacyclics of *T. vivax* IL3671 without feeder cell layers at 27 ^{O}C, and (2) transformation of the metacyclics to BSFs and continuous cultivation of the in vitro-transformed BSFs in the absence of feeder cells at 34 ^{O}C (Fig.1).

Step I. A total of 14 HMI media modified from HMI-93 medium (only HMI-107 medium shown in Table 1) were tested for their ability to support the growth of metacyclic-producing epimastigote populations in the absence of feeder cell layers. Bloodstream trypomastigotes obtained from infected bovine blood were suspended in each of the 14 media (10^7 BSFs/ml) and were placed in Plastek T-25 flasks (5 ml/flask) without feeder layers. The cultures were then maintained at 27 ^{O}C by replacing a part of the medium (1-2 ml/flask) 2-3 times a week. The BSFs maintained with HMI-107 medium in 2 flasks

transformed to epimastigotes and attached to the bottom of flasks within 12 days forming a number of small clusters. The clusters increased in number and size as the duration of culture was prolonged and covered the entire surface area by day 20. In contrast, BSFs maintained in other media were dead by day 14.

A total of 3 subcultures of the epimastigote populations were made on day 23 by the following procedures: (i) replacing the culture fluids with fresh medium (5 ml/flask), (ii) detaching epimastigote clusters by gentle scraping using a tip of Pasteur pipette, (iii) transfer of the epimastigote suspensions to new Plastek T-25 flasks (2-3 ml/flask) and addition of fresh medium (up to 5 ml/flask), (iv) keeping the flasks at 27 ^{O}C in a CO_2 incubator (Jouan EG115 IR, Saint-Herblain Cedex, France) for 1-2 h in the same manner described in System I, Step 3, and (v) maintaining the cultures at 27 ^{O}C by changing the medium (5 ml/flask) 2-3 times a week. In the subcultures, epimastigotes attached to the bottom of flasks within 24 h, formed small clusters and continued to increase in number and size (Fig. 6). The original flasks received fresh medium and were also maintained in the same manner. When the entire surface of flasks was covered with the epimastigote clusters, their density was reduced by gentle scraping. By repeating this procedure, epimastigote cultures were maintained up to 86 days in the same flasks. A small number of metacyclics emerged from epimastigote clusters which were first seen on day 14. Thereafter the clusters continued to produce metacyclics increasing in number until the cultures were terminated on day 86.

Step 2. Metacyclics were collected from the cultures prepared in Step 1 on days 25, 39 and 53 by the bovine plasma aggregation method, suspended in HMI-162 medium and then used to initiate BSF cultures without feeder cell layers. The metacyclic suspensions (4-6x10^6/ml) were placed in Plastek T-25 flasks (2.5 ml/flask, 2 flasks/group) and were incubated at 34 ^{O}C. Most metacyclics transformed to BSFs within 24 h and continued to proliferate, while some others formed small clumps and died within 14 days. In contrast to the BSFs of *T. vivax* IL 1392 in System I (Step 3), the majority of the BSFs of *T. vivax* IL 3671 were swimming freely in the medium and only very small numbers of the BSFs were loosely attached to the bottom of flasks. Thus, axenic cultures of IL 3671 BSFs were maintained by changing a part of the culture medium (0.5-1.5 ml/flask, depending on the density of BSFs) every 24 h.

Although HMI-162 medium supported the continuous growth of *T. vivax* IL 3671, the maximum density of the BSFs achieved in this medium was lower than 10^6ml (average 0.7x10^6/ml) and their population doubling times were longer than 18 h. Thus, an additional medium, HMI-163 (Table 1), was prepared from HMI-162 medium and was used to maintain the cultures on day 51 onwards in the BSF culture. The maximum density achieved in the modified medium was 1.8x10^6/ml and the shortest population doubling lime was 16.1 h. During the course of this study, a total of 50 subcultures were made from the cultures which were originally initiated from the epimastigote cultures on day 39. The subcultivation was carried out by transferring the culture fluids containing free swimming BSFs (0.5-1x10^6 BSFs/ml, 1 ml/flask) and fresh medium (1.5 ml/flask) to new flasks. The subcultures were maintained by changing the medium (1-1.5 ml/flask) every 24 h until the experiment was terminated on day 70 of the BSF culture. The pH of all the cultures in System II was always adjusted to 7.4 at the time of medium change and/or subcultivation as described in System I (Step 3).

System III was established in 1 step initiating the cultures with *G. m. centralis* proboscides infected with *T. vivax*, IL 3671 in the absence of feeder layer cells at 34 ^{O}C (Fig. 1).

Prior to the initiation, Vitrogen$^{(TM)}$ (purified bovine dermal collagen) (Collagen Co., Palo Alto, CA, USA)-coated plastic slips were prepared in Leighton tubes (Costar, Cat, No. 3393) according to the instructions given by the manufacturer of the Vitrogen. Infected proboscides were excised from tsetse flies and were cut into 2-3 pieces in a drop of HMI-162 medium. The pieces of proboscides (10-15 pieces made from 5 proboscides/flask) were placed on the surface of Vitrogen- coated plastic slips which were placed in 4 Plastek T-25 flasks containing HMI-162 medium (2.5 ml/flask), kept in a CO_2 incubator at 34 ^{O}C for 1-2 h in the same manner described above (System I, Step 3) and were then maintained at 34 ^{O}C.

During the initial 8 days, a small number of metacyclics emerged from the pieces of proboscides, and then rapidly transformed to free swimming BSFs in the medium and increased in number. During this period, a small volume of the medium (approximately 0.5 ml/flask) was changed every 48 h. On day 10, the Vitrogen-coated slips together with the pieces of proboscides were removed. During the next 10 days, the culture medium (0.5 ml/flask) was changed every 48 h. By day 20, population densities reached 4-6x10^5 BSFs/ml Thereafter the medium (0.5-1-5 ml/flask, depending on the density of BSFs) was changed every 24 h. A total of 42 subcultures were made in the same manner described in System II until the experiment was terminated on day 75, On day 59 onwards, the culture medium in this system was also changed from HMI-162 medium to HMI-163 medium (Fig. 7). The growth characteristics in HMI-163 medium were similar to those observed in the same medium in System II.

Electron microscopy

Transmission electron microscopy of *T. vivax* IL 1392 BSFs obtained from the axenic culture on day 61 in System I revealed the presence of an electron-opaque coat in all BSFs examined.

Infectivity

Two groups of 5 mice were inoculated (I.P.) with 10^6 BFSs/mouse of *T. vivax* IL 1392 obtained from the axenic cultures on days 19 (Group 1) and 98 (Group II) in System I, Step 3, respectively. Four of 5 mice in Group I developed infection within 3-5 days of inoculation, whereas 4 of 5 mice in Group II developed infection between 13 and 24 days after inoculation. Two of the 4 infected mice in Group I died by day 12, while 2 other infected mice in Group I and all the 4 infected mice in Group II showed chronic infection, and survived until the experiments were terminated on day 40. A young goat, aged 8 months, inoculated (I.V.) with 10^6 BSFs of *T. vivax* IL 1392 obtained on day 61 in System I, Step 3 also developed infection on day 11 after inoculation and died on day 74 after showing 9 successive waves of parasitaemia. Two Ayrshire calves, aged 6 months, were inoculated (I.V.) with 10^6 BSFs of *T. vivax* IL 3671 obtained from the axenic cultures on day 34 in System II and on day 53 in System III, respectively. Both animals developed infection by days 7 and 10 post-inoculation, and survived showing 5 and 3 successive waves of parasitaemia until the experiments were terminated on days 48 and 37, respectively.

DISCUSSION

The present study revealed that the "bloodstream form supporting factors" (bathocuproine sulfonate, L-cysteine, hypoxanthine, 2-mercaptoethanol, sodium pyruvate and thymidine) used for cultivating BSFs of *T. brucei* (Hirumi and Hirumi 1989) and *T. congolense* (Hirumi and Hirumi 1991) were also effective for the axenic cultivation of *T. vivax* BSFs. The study also demonstrated that L-cysteine and thymidine were essential factors for cultivating *T. vivax* BSFs without feeder layers as shown in the earlier work on the cultivation of *T. brucei* (Baltz et al. 1985; Duszenko et al. 1985; Hirumi and Hirumi 1989; Yabu et al. 1989) and *T. congolense* (Hirumi and Hirumi 1991), and that L-cysteine was effective in the presence of bathocuproine sulfonate (Hirumi and Hirumi 1989, 1991; Yabu et al. 1989; Zweygarth et al. 1991). However, an optimum concentration of each factor varied in supporting the best growth of not only different species (HMI-9 medium for *T. brucei* and HMI-93 medium for *T. congolense*) (Hirumi and Hirumi 1989, 1991) but also different stocks of a species (HMI-9 medium for IL 3000 and HMI-93 medium for 4 other stocks of *T. congolense*) (Hirumi and Hirumi 1991). In this study, a similar effect was observed with *T. vivax* BSFs. Neither HMI-9 medium nor HMI-93 medium supported the growth of *T. vivax* IL 1392, or of IL 3671, while HMI-162 medium modified from HMI-93 by changing the amounts of the factors supported the growth of IL 1392 but only poor growth of IL 3671. However, an additional modification (HMI-163 medium) of HMI-162 medium further improved the growth of IL 3671. These results suggest that if BSFs of new stocks of *T. vivax* could not adapt to the systems reported here, it would be advisable to establish optimum concentrations of the factors for the stocks. Particularly it would be important to establish an optimum amount of bathocuproine sulfonate since its effect to minimize toxic effects of hydrogen peroxide (Hirumi and Hirumi 1989, 1991; Yabu et al. 1989) produced by the autoxidation of

cysteine (Duszenko et al. 1985) varies among the reagents obtained from different suppliers (Yabu, Y., personal communication). In this study, as well as in our earlier work on *T. brucei* and *T. congolense* (Hirumi and Hirumi 1989, 1991), bathocuproinedisulfonic acid, disodium salt (2,9-dimethyl-4, 7-diphenyl-1, 10-phenanthrolinedisulfonic acid) obtained from a supplier (SIGMA, Cat. No.B-1125, Lot. 87F3794) was used.

Various mammalian sera have been used to supplement culture media (Baltz et al. 1985; Hirumi and Hirumi 1989, 1991; Zweygarth et al. 1991) including the media tested in this study. Our results obtained to date, including those obtained in the present study, indicate that young goat serum is the most suitable supplement for the axenic cultivation of BSFs of African trypanosomes, particularly of *T. congolense* (Hirumi and Hirumi 1991) and *T. vivax*.

Serum Plus(TM) was used as a supplement in the series of HMI media in the present study as well as in the earliers work on *T. brucei* and *T. congolense*. According to the manufacturer, it contains a low level of fetal bovine serum proteins (13 μg/ml), specific growth promoting factors, transport proteins, hormones, a high level of glucose (12.24 μg/ml), and low amounts of hemoglobin (10 mg/ml) and endotoxin (5.8 ng/ml) with a pH of 7.32 and osmolarity of 378 mOsm. Although further details of the contents have not been released by the manufacturer, Serum Plus supplement appeared to be effective in media used for stocks which were difficult to adapt to their axenic culture conditions.

Two basal media. Eagle's MEM (Baltz et al. 1985; Duszenko et al. 1985; Zweygarth et al. 1991) and Iscove's DMEM (Hirumi and Hirumi 1989, 1991; Yabu et al. 1989) which is a modification of Dulbecco's modified MEM, have been used in the earlier work. In our earlier study, BSFs of *T. brucei* and *T. congolense* grew better in DMEM-based media than in MEM-based media, while insect stages of *T. congolense* grew better in the latter media. In the present study, DMEM-based media supported the continuous growth of both insect stages (HMI-107 medium containing praline) and BSFs (HMI-162 and HMI-163 media) of *T. vivax* in the absence of feeder cell layers, while the insect stages, as well as the BSFs, continued to grow in MEM-based media (TVM-1 and TVM-22 media) in the presence of feeder layers. These results suggests that DMEM is a more suitable basal medium than MEM for the axenic cultivation of BSFs of the 3 trypanosome species. This could be one of the possible reasons for differing results on the maximum densities and the shortest population doubling times of the same stock (IL 1392) between that obtained in the present study (maximum density: $3.5x10^6$/ml; shortest population doubling time: 13.5 h) and that obtained in an earlier work (maximum density: $5x10^5$/ml; shortest population doubling time: 21 h) (Zweygarth et al. 1991).

Most stocks of *T. vivax* are infective for ruminants but not for rodents, except a few stocks including IL 1392 (Gathuo et al. 1987). In our earlier studies, infectivities of metacyclic populations of *T. vivax* IL 1392 produced in the presence of feeder cell layers became lower for mice but remained unchanged for cattle and goats as the duration in culture was prolonged (unpublished data). Although no extensive tests on the infectivity of *T. vivax* BSFs propagated in the axenic culture systems were carried out in the present study, the results obtained with IL 1392 BSFs in mice suggested that the virulence of the stock for rodent hosts might also be altered in the long-term culture. However, further studies are required to clarify the mechanisms that regulate the virulence of African trypanosomes. The axenic culture systems reported here may facilitate such studies.

Furthermore a preliminary study indicated that the axenic culture systems developed in this study were suitable for investigating the sensitivity of *T. vivax* BSFs to trypanocidal drugs (Kaminsky, R., personal communication).

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