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Trypanosoma brucei gambiense: HMI-9 medium containing methylcellulose and human serum supports the continuous axenic *in vitro* propagation of the bloodstream form

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ABSTRACT

Trypanosoma brucei (*T.b.*) *gambiense* causes the chronic form of human African trypanosomiasis or sleeping sickness. One of the major problems with studying *T.b. gambiense* is the difficulty to isolate it from its original host and the difficult adaptation to *in vivo* and *in vitro* mass propagation. The objective of this study was to evaluate if an established method for axenic culture of pleomorphic bloodstream form *T.b. brucei* strains, based on methylcellulose containing HMI-9 medium, also facilitated the continuous *in vitro* propagation of other bloodstream form *Trypanozoon* strains, in particular of *T.b. gambiense*. Bloodstream form trypanosomes from one *T.b. brucei*, two *T.b. rhodesiense*, one *T. evansi* and seven *T.b. gambiense* strains were isolated from mouse blood and each was concurrently cultivated in liquid and methylcellulose-containing HMI-9 based medium, either with or without additional human serum supplementation, for over 10 consecutive sub passages. Although HMI-9 based medium supplemented with 1.1% (w/v) methylcellulose supported the continuous cultivation of all non-*gambiense* strains better than liquid media could, the *in vitro* cultivation of all *gambiense* strains was only achieved in HMI-9 based medium containing 1.1% (w/v) methylcellulose, 15% (v/v) fetal calf serum and 5% (v/v) heat-inactivated human serum.

1. Introduction

Trypanosoma brucei gambiense causes the chronic form of human African trypanosomiasis or sleeping sickness, one of the most neglected diseases affecting rural populations in sub-Saharan Africa (Simarro et al., 2008). Although very closely related to *T.b. brucei* and *T.b. rhodesiense*, this parasite subspecies has its peculiarities of which the underlying mechanisms remain unknown, for example its restricted host range, its consistent normal human serum resistance and its differential virulence in human and laboratory rodents (Gibson, 1986). One of the major problems with studying *T.b. gambiense* is the difficulty to isolate it from its original host and the difficult adaptation to *in vivo* and *in vitro* mass propagation.

Direct isolation from the tsetse fly and the mammalian host has been achieved using the Kit for *In Vitro* Isolation (KIVI) that supports the growth of procyclic trypomastigotes (Aerts et al., 1992). However, KIVI is selective for certain subpopulations of *T.b. gambiense* (Jamonneau et al., 2003). To obtain the bloodstream form trypomastigotes from procyclic populations, the trypanosomes have to be passed through tsetse flies and trypanosome susceptible rodents, a particularly cumbersome manipulation with low success rate (Ravel et al., 2006). Thus, to produce sufficient quantities of bloodstream forms, it is preferred to isolate and propagate *T.b. gambiense* bloodstream forms immediately, either *in vivo* through rodent inoculation or via *in vitro* culture.

On isolation of bloodstream form *T.b. gambiense* in rodents, some recent improvements have been made. Büscher and co-workers reported higher isolation success rates in the thicket rat *Grammomys surdaster* than in *Mastomys natalensis* or immunosuppressed laboratory mice or rats (Büscher et al., 2005). Yet, in most instances

Abbreviations: HMI-9, Hirumi's modified Iscove's medium 9; HF, HMI-9 without Serum Plus but with 15% (v/v) of fetal calf serum; HH, HF with an additional 5% (v/v) heat-inactivated human serum; HFM, HF with 1.1% (w/v) methylcellulose; HHM, HH with 1.1% (w/v) methylcellulose.

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it is not feasible to bring these rodents close enough to place of diagnosis or treatment to allow direct inoculation of the patient's specimen. This problem has been solved with the introduction of a new cryomedium for *in situ* cryopreservation of blood from *gambiense* patients prior to inoculation of rodents in the laboratory (Maina et al., 2007).

For adaptation of *T. brucei* bloodstream form trypomastigotes to *in vitro* culture, diverse methods have been described, mostly making use of various combinations of media ingredients and animal sera and of feeder layer cells during the initial passages (Hirumi et al., 1977; Duszenko et al., 1985; Baltz et al., 1985). Hirumi and Hirumi succeeded in formulating an axenic medium for continuous cultivation of *T. brucei* (Hirumi's modified Iscove's medium, HMI-9) but adaptation to this medium invariably induces a period of massive cell death and hence a selection can occur before a continuous culture is established (Hirumi and Hirumi, 1989). In contrast with what is observed in liquid axenic cultures, primary cultures established using solid HMI-9-agarose plates do not suffer from massive cell death at the initiation stage (Vassella and Boshart, 1996).

Vassella and co-workers described that HMI-9 based medium containing low melting point agarose or methylcellulose, providing a high molecular weight matrix, allows several pleomorphic *T.b. brucei* strains to proliferate without aberrant cell division or growth arrest. Apart from culture and cryostabilisation, these media are also compatible with transfection of bloodstream form trypomastigotes (Vassella et al., 2001; McCulloch et al., 2004). So far, experiments on axenic *in vitro* culture in media containing methylcellulose have not yet been conducted with *T.b. gambiense*.

We investigated the suitability of HMI-9 based medium containing methylcellulose for the continuous axenic *in vitro* propagation of bloodstream form *T.b. gambiense* strains. In addition, we evaluated whether supplementation of this viscous culture medium with human serum improved the *in vitro* propagation of *T.b. gambiense* strains further.

2. Methods

2.1. Ethics statement

All experiments on animals were approved by the Institute of Tropical Medicine's Animal Ethics Committee under license PAR010-PB-MR-NEUROTRYP.

2.2. Culture media

Iscove's modified Dulbecco's medium powder and fetal calf serum (heat-inactivated EU origin) were purchased from Invitrogen. Methylcellulose (5140 mPa S) was purchased from Fluka. All other ingredients were from Sigma–Aldrich. A recipe to prepare a concentrated HMI stock solution, without serum, was adapted from McCulloch (McCulloch et al., 2004). This HMI stock solution was used to prepare four different culture media (Table 1). HMI – fetal calf serum (HF) is a close variant of HMI-9 but without Serum Plus and with a higher concentration (15% v/v) of fetal calf serum (FCS). HMI – fetal calf serum – methylcellulose (HFM) is HF containing a final concentration of 1.1% methylcellulose. HMI-human serum (HH) contains (15% v/v) FCS and 5% (v/v) heat-inactivated human serum. HMI – human serum – methylcellulose (HHM) is HH containing a final concentration of 1.1% (w/v) methylcellulose. The final concentration of methylcellulose in HFM and HHM medium was obtained by mixing the concentrated HMI stock solution with a 3% (w/v) methylcellulose solution. This concentrated methylcellulose solution was prepared in distilled water at 65 °C under continuous stirring followed by steam sterilisation for 20 min at 120 °C and rapid cooling in an ice bath under vigorous magnetic stirring (McCulloch et al., 2004).

Table 1
Recipe for 1 l of each of the HMI-9 variations[a].

Ingredient	HF	HH	HFM	HHM
2 × Iscove's modified Dulbecco's medium	365	365	365	365
100 mM hypoxanthine in 0.1 N NaOH	10	10	10	10
5 mM bathocuproine disulphonic acid	10	10	10	10
20 mM β-mercaptoethanol	10	10	10	10
16 mM thymidine	10	10	10	10
100 mM pyruvate	10	10	10	10
100 mM cysteine	10	10	10	10
Fetal calf serum	150	150	150	150
Human serum	0	50	0	50
H_2O	425	375	50	0
3% (w/v) methylcellulose	0	0	375	375

[a] All ingredients are given in milliliter per liter.

2.3. Trypanosomes

One *T.b. brucei*, two *T.b. rhodesiense*, one *T. evansi* and seven *T.b. gambiense* strains were used for the *in vitro* culture experiments. Original host, country and year of isolation and number of passages in rodents are given in Table 2. All *T.b. rhodesiense* and *T.b. gambiense* stocks originated from patients.

To obtain a population of bloodstream form trypomastigotes, female OF-1 mice (Charles River, Belgium), immune suppressed with 200 mg/kg cyclophosphamide (Endoxan, Baxter) 24–48 h before infection, were infected intraperitoneally with $1–10 \times 10^6$, parasites from a cryostabilate diluted 1:1 in phosphate buffered saline glucose pH 8.0 (PSG) (Lanham and Godfrey, 1970). The matching method was used to monitor parasitemia in tail-blood (Herbert and Lumsden, 1976). At first peak parasitemia, when the blood contained $1–10 \times 10^6$ ml^{-1} long slender bloodstream form trypomastigotes, the mouse was anaesthetised and its chest was disinfected with 70% ethanol. Blood was collected by cardiac puncture with a heparinised 1 ml syringe, transferred in a 15 ml tube near an open flame and centrifuged at 1000*g* for 15 min at 25 °C to concentrate the trypanosomes in the buffy coat. After centrifugation, the buffy coat was gently resuspended in the overlaying plasma, taking care not to disturb the red blood cell sediment. This parasite suspension in plasma was used to initiate *in vitro* cultures.

2.4. In vitro culture

The suspension was transferred to an equal volume of HF and the parasites were counted and diluted to a concentration of 1×10^5 cells ml^{-1}. Several primary cultures were initiated at 1×10^4 cells ml^{-1} by inoculating 50 μl of this suspension in 450 μl of medium in a 24-well plate. Cultures were incubated at 37 °C and 5% CO_2 and monitored every 24 h by phase contrast inverted microscopy or counting in disposable counting chambers (Uriglass, Menarini). To establish continuous cultures, primary cultures showing a density increase in the range of $1–10 \times 10^5$ cells ml^{-1} were sub passaged to densities of $1–10 \times 10^4$ cells ml^{-1} using at least 80–90% fresh medium. A culture was considered continuous when 10 consecutive sub passages could be made.

2.5. Cryostabilisation

Adapted cultures were scaled up tenfold in a 6-well plate format by inoculating 4.5 ml fresh medium with 500 μl of an exponentially growing culture. When the parasite density reached 1×10^6 cells ml^{-1}, 5 ml of the culture was diluted with 20 ml