LITERATURE CITED

GLEASON, L. N. 1987. Population dynamics of *Pomphorhynchus bulbocolli* in *Gammarus pseudolimnaeus*. Journal of Parasitology **73:** 1099–1101.

JENSEN, T. 1952. The life-cycle of the fish acanthocephalan, *Pomphorhynchus bulbocolli* (Linkins) Van Cleave 1919, with some observations on larval development in vitro. Ph.D. Dissertation. University of Minnesota, Minneapolis, 74 p.

MUZZALL, P. M. 1982. Parasites of *Gammarus pseudolimnaeus* and *Hyallela azteca* (Crustacea: Amphipoda) in three south-central Michigan localities. Proceedings of the Helminthological Society of Washington **49:** 289–294.

NICKOL, B. B., AND G. E. DAPPEN. 1982. *Armadillidium vulgare* (Isopoda) as an intermediate host of *Plagiorhynchus cylindraceus* (Acanthocephala) and isopod response to infection. Journal of Parasitology **68:** 570–575.

J. Parasitol., 75(6), 1989, p. 985–989

Continuous Cultivation of *Trypanosoma brucei* Blood Stream Forms in a Medium Containing a Low Concentration of Serum Protein Without Feeder Cell Layers

Hiroyuki Hirumi and Kazuko Hirumi, International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi, Kenya

ABSTRACT: Blood stream forms (BSF) of *Trypanosoma brucei brucei* GUTat3.1 were propagated in vitro in the absence of feeder layer cells at 37 C, using a modified Iscove's medium (HMI-18). The medium was supplemented with 0.05 mM bathocuproine sulfonate, 1.5 mM L-cysteine, 1 mM hypoxanthine, 0.2 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.16 mM thymidine, and 20% (v/v) Serum Plus® (SP) (Hazleton Biologics, Lenexa, Kansas). The latter contained a low level of serum proteins (13 μg/ml). Each primary culture was initiated by placing 3.5–4 × 10^6 BSFs isolated from infected mice in a flask containing 5 ml of the medium (HMI-9) supplemented with 10% fetal bovine serum (FBS) and 10% SP. The cultures were maintained by replacing the medium every 24 hr for 5–7 days. During this period, many BSFs died. However, from day 4 onward, long slender BSFs increased in number. On days 5–7, trypanosome suspensions were pooled and cell debris was removed by means of diethylaminoethyl cellulose (DE52) column chromatography. Blood stream forms then were collected by centrifugation, resuspended in fresh medium at 7–9 × 10^5/ml, and transferred to new flasks. Subcultures were maintained by readjusting the BSF density to 7–9 × 10^5/ml every 24 hr. Concentrations of FBS were reduced gradually at 5–7-day intervals by alternating the amounts of FBS and SP in HMI-9 with 5% FBS and 15% SP, with 2% FBS and 18% SP, and finally with 20% SP (HMI-18). By this method, 2–3 × 10^6 BSFs/ml were obtained consistently every 24 hr, for more than 80 days. This system also has been applied successfully to the cultivation of BSFs of *Trypanosoma brucei gambiense* IL2343.

The development of in vitro techniques for cultivating African trypanosomes may be divided into 3 phases. Phase I, from 1903 to 1976; during this period only uninfective procyclic forms of *Trypanosoma brucei* and *Trypanosoma congolense* were cultivated continuously (Evans, 1978). Phase II, from 1977 to 1988; during this period basic techniques were developed for cultivating all developmental stages of the 3 major pathogens causing African trypanosomiasis, namely *T. brucei, T. congolense,* and *Trypanosoma vivax.* Most of the stocks and clones tested to date were cultivated successfully (Hirumi et al., 1980; Brun and Jenni, 1987; Gray et al., 1987). Phase III is presently underway. The main objectives in Phase III are the development of a mammalian serum-independent culture system; a feeder layer-free system, particularly for cultivating blood stream forms (BSFs) of the parasites; and a combination of both. If such systems become available reliably, they will facilitate investigations of factors that promote growth of the parasites and regulate their stage differentiations, and of mechanisms that underly parasite resistance to trypanocidal drugs. Along these lines, Baltz et al. (1985) demonstrated that BSFs of 10 stocks of the *T. brucei* subgroup could be cultivated in a feeder layer-free system using a modified Eagle's minimum essential medium (MEM) that was supplemented with 10% (v/v) mammalian serum derived from various animals. In that system, BSFs were maintained initially in

the presence of a macrophage feeder layer during an "initiation stage" and then in a subsequent "adaptation stage." Duszenko et al. (1985) also reported that L-cysteine is 1 of the essential factors to support the growth of BSFs of *T. brucei* MTIat1.4 and that the BSFs can be subcultured continuously and axenically by adding cysteine at regular intervals (twice a day) and in appropriate concentrations in a modified MEM. The present paper reports an improved method that supports the continuous propagation of BSFs of *T. b. brucei* GUTat3.1 in a relatively simple manner for more than 80 days in the absence of feeder cell layers, using a medium containing a low concentration of serum proteins.

Trypanosoma b. brucei GUTat3.1 is a cloned derivative of a stock (EVE 10: Edinburgh Veterinary Expedition) that was isolated originally in 1966 from a naturally infected bovine in Uganda (Onyango et al., 1966). Eighteen culture media, HMI-1 to HMI-18 (listed in Table I) were tested. These were modified either from MEM (GIBCO, Scotland), Leibovitz's L-15 medium (GIBCO), or from Iscove's modified Dulbecco's MEM (GIBCO) by supplementing with various amounts of bathocuproine sulfonate, L-cysteine, hypoxanthine, 2-mercaptoethanol, sodium pyruvate, thymidine, and heat-inactivated fetal bovine serum (FBS) (Flow Laboratories, Scotland) and/or Serum Plus® (SP) (Hazleton Biologics, Lenexa, Kansas). According to the manufacturer, the latter (lot no. 14001004) contained a low level of FBS proteins (13 μg/ml), specific growth promoting factors, transport proteins, hormones, a high level of glucose (12.24 mg/ml), and low amounts of hemoglobin (10 μg/ml) and endotoxin (5.8 ng/ml) with a pH of 7.32 and osmolarity of 378 mOsm. Blood stream forms were isolated from GUTat3.1-infected Balb/c mouse blood by suspending the blood in each culture medium tested (1 part of the blood and 9 parts of the medium), centrifuging at 200 *g* for 5 min at 4 C, transferring BSF-containing supernatants to 25-cm^2 T-type flasks (Costar, Cambridge, Massachusetts), keeping the flasks for 2–3 hr at 37 C to settle small amounts of remaining blood cells on the bottom of the flasks and then collecting the BSF-containing medium from the flasks by gentle pipetting. Concentrations of BSFs were adjusted by adding appropriate volumes of the medium. In all experimental groups, each primary culture was initiated by placing 3.5–4.0 $\times 10^6$ BSFs in a flask containing 5 ml of 1 of the media. Flasks were maintained at 37 C by replacing 2–3 ml of the medium every 24 hr for 5–7 days. During this period, many BSFs died. However, from day 4 onward, long slender BSFs increased in number in cultures with appropriate media. On days 5–7, trypanosome suspensions in such cultures were pooled, generally from 4 primary cultures in each experimental group, and cell debris was removed by means of diethylaminoethyl cellulose (DE52; Whatman, England) column chromatography (Lanham and Godfrey, 1970). Blood stream forms were then collected by centrifugation at 1,630 *g* for 10 min at 4 C, resuspended in fresh medium at different BSF densities, and subcultured in new flasks (5 ml/flask). Subcultures were maintained at 37 C by replacing 3.0–3.8 ml of the medium, and readjusting the BSF density every 24 hr as described below. Caps of culture flasks were kept loose so that the media were exposed to 2% CO_2 in air in a CO_2-incubator for 3–4 hr immediately after subcultivation. Then they were tightly closed. The improvement in this system was carried out in 4 steps.

Step I: To establish an optimal basic medium for initiating the primary culture, 15 modified media (HMI-1 to HMI-13, HMI-15, and HMI-16) were tested initially in primary cultures (4 flasks/medium). Many trypanosomes transformed to stumpy forms but subsequently died during the primary cultivation. By day 8, no trypanosome was seen in cultures that were maintained with HMI-1 to HMI-6, HMI-12, HMI-13, HMI-15, and HMI-16. However, in cultures with HMI-7, and HMI-9 to HMI-11, long slender BSFs increased in number from day 4 onward. Of these 4 media, HMI-9 supported the best growth of long slender BSFs. These 4 media were tested further in subcultures (4 flasks/group) that were prepared by placing 7 $\times 10^5$ BSF/ml in new flasks (5 ml/flask) after the DE52 column separation. They were maintained by replacing 1.7 ml of the medium every 24 hr for 5 days. Results of 1 such test are shown in Figure 1A (a, b), indicating that the growth rates of BSFs in HMI-9 were higher than those in HMI-7, modified from MEM with the same amounts of all the supplements used in HMI-9. Similar results were obtained in the other 2 media. Thus, HMI-9 was used as the initiation medium as well as the control medium for further improvements.

Step II: Although HMI-9 supported the best growth of BSFs among the 15 media tested in step I, the yields of BSFs declined after day 3. A similar growth pattern of *T. brucei* BSFs also was

TABLE I. Modified culture media tested for cultivating *Trypanosoma brucei brucei* blood stream forms and trypanosome growth in primary cultures.

Medium code number	Basic medium*			Supplements†										Growth in primary culture‡
				Serum (%, v/v)				Other (mM)						
	MEM	L-15	IMDM	FBS	SP	2WCS	6MCS	BAC	CYS	HYP	MER	PYR	THY	
HMI-1	1/2	1/2		20										−
HMI-2	1/2	1/2		20				0.03	0.3		0.1			−
HMI-3	1/2	1/2		20				0.03	0.3	1.0	0.1	2		−
HMI-4	1/2	1/2		20				0.03	0.3	0.5	0.1	2		−
HMI-5	1			20				0.05	1.5	1.0	0.1	2		−
HMI-6	1			10	10			0.05	1.5	1.0	0.1	1		−
HMI-7	1			10	10			0.05	1.5	1.0	0.2	2	0.16	+
HMI-8	1			5	15			0.05	1.5	1.0	0.2	1	0.16	−
HMI-9			1	10	10			0.05	1.5	1.0	0.2	1 (2)§	0.16	+
HMI-10			1	5	15			0.05	1.5	1.0	0.2	1 (2)	0.16	+
HMI-11			1	10				0.05	1.5	1.0	0.2	1 (2)	0.16	+
HMI-12			1	10	10			0.05	1.5	1.0	0.2	(1)	0.16	−
HMI-13			1	5	15			0.05	1.5	1.0	0.2	(1)	0.16	−
HMI-14			1	2	18			0.05	1.5	1.0	0.2	1 (2)	0.16	NT
HMI-15			1	10		10		0.05	1.5	1.0	0.2	1 (2)	0.16	−
HMI-16			1		10		10	0.05	1.5	1.0	0.2	1 (2)		−
HMI-17			1		10			0.05	1.5	1.0	0.2	1 (2)	0.16	NT
HMI-18			1		20			0.05	1.5	1.0	0.2	1 (2)	0.16	NT

* MEM, Eagle's minimum essential medium; L-15, Leibovitz's L-15 medium; IMDM, Iscove's modified Dulbecco's MEM.

† FBS, fetal bovine serum; SP, Serum Plus®; 2WCS, 2-wk-old calf serum; 6MCS, 6-mo-old calf serum; BAC, bathocuproine sulfonate; CYS, L-cysteine; HPY, hypoxanthine; MER, 2-mercaptoethanol; PYR, sodium pyruvate; THY, thymidine; 1/2, 2 media were mixed (1:1); 1, 1 basic medium was used.

‡ Primary cultures were maintained by replacing 2–3 ml of the medium every 24 hr; (−), no trypanosome was seen by day 8; (+), trypanosomes continued to grow over 8 days; NT, not tested in primary cultures, but used in subcultures for further improvements.

§ Final concentration in ( ); the original basic medium contained 1 mM of sodium pyruvate.

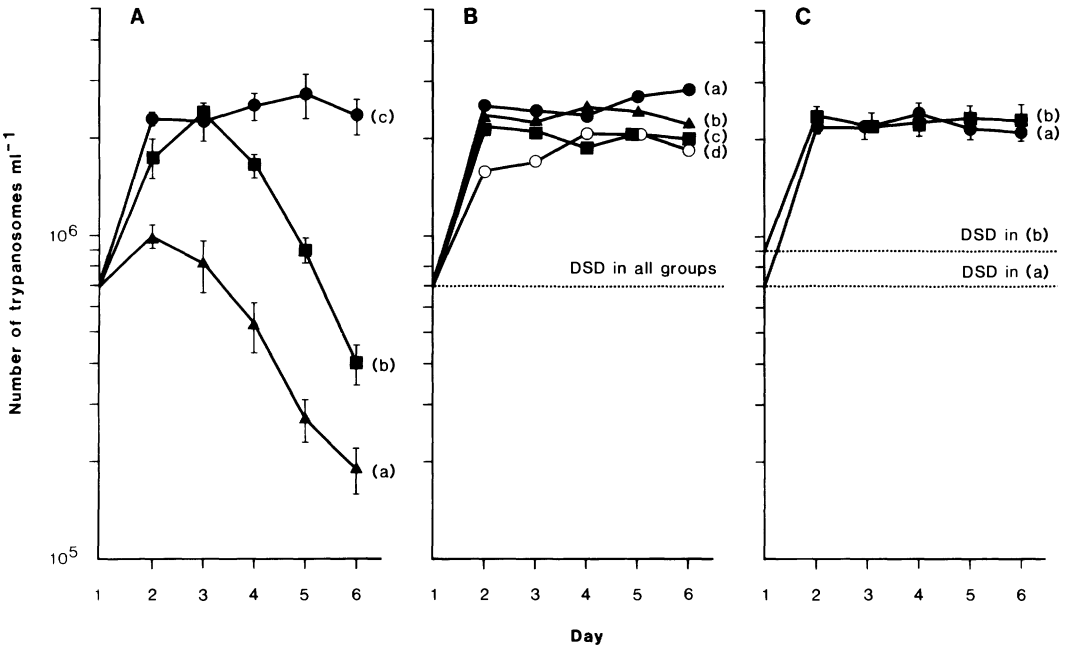

FIGURE 1. Yields of blood stream forms (BSFs) of *Trypanosoma brucei brucei* GUTat3.1 propagated at 37 C under various culture conditions in the absence of feeder cell layers. Data expressed as the mean of 4 replicates ± SD in (A, C); SDs $< \pm 0.38$ in (B). **A.** BSF populations maintained either in HMI-7 medium (a) or in HMI-9 medium (b, c), by diluting the population density to ⅔ (a, b) or by readjusting to 7×10^5 BSFs/ml (c) every 24 hr. **B.** BSF populations maintained by readjusting the daily seeding density (DSD) to 7×10^5 BSFs/ml every 24 hr in HMI-9 supplemented with 10% (v/v) fetal bovine serum (FBS) and 10% (v/v) Serum Plus® (SP) (a), HMI-10 medium with 5% FBS and 15% SP (b), HMI-14 medium with 2% FBS and 18% SP (c), and HMI-18 medium with 20% SP (d). **C.** BSF populations maintained in HMI-9 medium by adjusting the DSD to 7×10^5 BSFs/ml (a), and in HMI-18 medium to 9×10^5 BSFs/ml (b).

observed earlier (Hirumi et al., 1980) in the presence of feeder cell layers and was improved by adjusting the daily seeding density (DSD). In step II, various DSDs, therefore, were examined in subcultures (4 flasks/group) for 5 days using HMI-9. It was established that consistent yields of 2–3 $\times$ 10^6 BSFs/ml could be obtained by seeding the subcultures with 7×10^5 BSFs/ml every 24 hr (Fig. 1A, c).

Step III: To minimize the amount of FBS in culture medium, concentrations of FBS were gradually reduced at 5–7-day intervals by alternating the amounts of FBS (10%) and SP (10%) in HMI-9 with 5% FBS and 15% SP (HMI-10), with 2% FBS and 18% SP (HMI-14), and finally with 20% SP (HMI-18). After being adapted to these media, the growth rates of BSFs in each medium were compared by seeding subcultures (4 flasks/group) with 7×10^5 BSFs/ml every 24 hr for 5 days. The results demonstrated that almost consistent daily yields could be obtained in all media during this period, although the yields in HMI-18 (1.6–2.1 $\times$ 10^6 BSFs/ml; Fig. 1B, d) were slightly lower than those (1.9–3.0 $\times$ 10^6 BSFs/ml; Fig. 1B, a–c) obtained in the other 3 media.

Step IV: To retain consistent daily yields of above 2×10^6 BSFs/ml in HMI-18, DSD was readjusted to 9×10^5 BSFs/ml, and the growth rates were compared with those readjusted at DSD 7×10^5/ml in HMI-9 for 5 days (4 flasks/group). The readjusted population in HMI-18 (Fig. 1C, a) consistently produced BSFs at the same level (2.0–2.5 $\times$ 10^6/ml) as obtained in HMI-9 (Fig. 1C, b).

Blood stream forms also could be cultivated with HMI-18 in 24-well culture plates (Costar) with 2% CO_2 in air using a similar method to that employed in the flask cultures.

Trypanosomes propagated in subcultures retained the morphological characteristics of long slender BSFs seen in the infected mouse blood, and those harvested on day 71 (56 days in HMI-9, HMI-10, and HMI-14, and 14 days in HMI-18) were covered with an electron-opaque surface coat as examined by transmission electron

microscopy. They gave rise to infections in all mice, which were inoculated intraperitoneally with tenfold dilutions of suspensions, containing 5×10^3 to 5×10 such trypanosomes (4 mice/group), within 10 days after inoculation. In the group inoculated with 5 BSFs/mouse, 3 of 4 mice became infected. The same results were obtained with trypanosomes harvested on day 84. The subcultures were maintained in this system until the experiment was terminated on day 86.

This system also has been applied successfully to the cultivation of BSFs of *Trypanosoma brucei gambiense* IL2343, a derivative of a clone STIB386AA that was isolated originally in 1978 from a patient in the Ivory Coast (Jenni et al., 1986).

The present study confirmed the findings in the earlier work by Baltz et al. (1985) that indicated a requirement for hypoxanthine, sodium pyruvate, and thymidine for cultivating *T. brucei* BSFs in a feeder layer-free system, although a higher amount of hypoxanthine (10 times higher than in Baltz's medium B) was found to be optimal in HMI-18. Although the original Iscove's modified Dulbecco's MEM contained 1 mM of sodium pyruvate, a final concentration of 2 mM was required to support the growth of BSFs during the primary cultivation even though BSFs could be maintained in subculture without such addition.

Duszenko et al. (1985) demonstrated that L-cysteine is an essential factor for the cultivation of BSFs of *T. brucei* axenically. To minimize toxic effects of hydrogen peroxide produced by the autoxidation of L-cysteine, they added freshly prepared L-cysteine solution to the culture medium twice a day at appropriate concentrations, depending on the density of BSFs at the time of medium change. In the present study it was found also that L-cysteine is an essential supplement in the HMI media. Furthermore, the addition of L-cysteine was simplified by adding bathocuproine sulfonate (a chelating agent) and by regulating the DSDs at the standardized levels (7–9×10^5 BSF/ml). In this way, the final medium, containing L-cysteine, could be used for at least 10 days after its preparation (the maximum shelf time has not been established) and 1 medium change was sufficient to produce $2–3 \times 10^6$ BSF/ml every 24 hr. The chelating agent has been used earlier by Tegoshi (1988) in a modified MEM containing L-cysteine for the cultivation of *Pneumocystis carinii* in a feeder layer-free system.

The final maintenance medium (HMI-18) developed in this study still contained a low level of FBS proteins (13 μg/ml) in SP. Thus, further improvement will be needed to establish a completely serum-independent culture system. Nevertheless, differing results that often have been due to variability between FBS batches have been minimized by using HMI-18 containing no additional FBS.

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LITERATURE CITED

BALTZ, T., D. BALTZ, CH. GIROUD, AND J. CROCKETT. 1985. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense*. EMBO Journal **4:** 1273–1277.

BRUN, R., AND L. JENNI. 1987. Salivarian trypanosomes: Bloodstream forms. *In* In vitro methods for parasite cultivation, A. E. R. Taylor and J. R. Baker (eds.). Academic Press, Inc., New York, p. 94–117.

DUSZENKO, M., M. A. J. FERGUSON, G. S. LAMONT, M. R. RIFKIN, AND G. A. M. CROSS. 1985. Cysteine eliminates the feeder cell requirement for cultivation of *Trypanosoma brucei* bloodstream forms in vitro. Journal of Experimental Medicine **162:** 1256–1263.

EVANS, D. A. 1978. Kinetoplastida. *In* Methods in cultivating parasites in vitro, A. E. R. Taylor and J. R. Baker (eds.). Academic Press, Inc., New York, p. 55–88.

GRAY, M. A., H. HIRUMI, AND P. R. GARDINER. 1987. Salivarian trypanosomes: Insect forms. *In* In vitro methods for parasite cultivation, A. E. R. Taylor and J. R. Baker (eds.). Academic Press, Inc., New York, p. 118–152.

HIRUMI, H., K. HIRUMI, R. T. NELSON, AND J. J. BWAYO. 1980. Present status of the cultivation of African trypanosomes. *In* Tropical diseases research, Series 3 (WHO). Schwabe & Co. AG, Basel, p. 165–200.

JENNI, L., S. MARTI, J. SCHWEIZER, B. BETSCHART, R. W. F. LE PAGE, J. M. WELLS, A. TAIT, P. PAINDAVOINE, E. PAYS, AND M. STEINERT. 1986. Hybrid formation between African trypanosomes during cyclical transmission. Nature **322:** 173–175.

LANHAM, S. M., AND D. G. GODFREY. 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Experimental Parasitology **28:** 521–534.

ONYANGO, R. J., K. VAN HOEVE, AND P. DE RAADT. 1966. The epidemiology of *Trypanosoma rhodesiense* sleeping sickness in Alego location, Central Nyanza, Kenya. 1. Evidence that cattle may act as reservoir hosts of trypanosomes infective to man. Transactions of the Royal Society of Tropical Medicine and Hygiene **60:** 175–182.

TEGOSHI, T. 1988. New system of in vitro cultivation of *Pneumocystis carinii* without feeder cells. Journal of Kyoto Prefectural University of Medicine **97:** 1473–1482.