Acad. Sci. U.S.A. **73**, 1427 (1976); R. Vigneri, I. D. Goldfine, K. Y. Wong, G. J. Smith, V. Pezzino, *J. Biol. Chem.* **253**, 2098 (1978).
10. I. D. Goldfine, R. Vigneri, D. Cohen, N. B. Pliam, *Nature (London)* **269**, 698 (1977).
11. C. R. Kahn, *J. Cell Biol.* **70**, 261 (1976).
12. J. J. M. Bergeron, W. H. Evans, I. I. Geschwind, *ibid.* **59**, 771 (1973).
13. I. D. Goldfine, *Life Sci.*, in press; R. Vigneri and I. D. Goldfine, *Minerva Endocrinol.* **2**, 59 (1977); I. D. Goldfine, *Receptors in Pharmacology* (Dekker, New York, 1978), pp. 335-377; *Diabetes* **26**, 148 (1977).
14. This investigation was supported by the Medical Research Service of the Veterans Administration and by grant AM-19415 from NIH.

8 May 1978; revised 17 July 1978

Growth of Infective Forms of *Trypanosoma rhodesiense* in vitro, the Causative Agent of African Trypanosomiasis

Abstract. *A new approach to the culture of African trypanosomes led to the growth of the infective forms of the causative agent of human African trypanosomiasis. Infective cultures of* Trypanosoma rhodesiense *were initiated and maintained in vitro on Chinese hamster lung cells. By changing daily one-third of the Hepes-buffered RPMI 1640 medium containing 20 percent fetal bovine serum, the trypanosome numbers increased to 3 × 10⁶ to 5 × 10⁶ cells per milliliter. After 80 days in vitro at 37°C, the cultured trypomastigotes are infective for mice and rats and morphologically similar to bloodstream trypomastigotes in having a subterminal kinetoplast and a surface coat. In addition, they possess* L-α-*glycerophosphate oxidase, the predominant steady-state terminal oxidase of bloodstream trypomastigotes.*

Trypanosomiasis has been placed by the World Health Organization high on the list of the ten major health problems facing mankind today. Sleeping sickness constitutes a permanent and serious risk to the health and well-being of at least 35 million people. Biochemical and immunological studies of *Trypanosoma rhodesiense* and *Trypanosoma gambiense*, the causative agents of the disease in humans, would be facilitated if the infective stages of these organisms could be grown in vitro.

The development of a medium and conditions under which infective trypanosomes can be maintained in vitro has been attempted for numerous years (*1*), and the descriptions by Hirumi *et al.* (*2*) of conditions that will allow the development of infective forms of *Trypanosoma brucei* in culture have stimulated further studies in this area. Using bovine fibroblast-like cells in Hepes-buffered RPMI 1640 medium (*3*) containing 20 percent inactivated fetal bovine serum (FBS), Hirumi *et al.* were able to grow trypanosomes that were infective for mammalian hosts and also retained the morphological characteristics of long, slender bloodstream trypomastigotes with the surface coat (*2*).

Our studies were directed toward identifying established tissue culture cell lines that would support the growth of *T. rhodesiense* at high yield and toward developing simple procedures for establishing infective trypomastigotes in culture. In addition, we wanted to determine the biochemical similarities between *Trypanosoma rhodesiense* infective trypomastigotes maintained in culture and the slender bloodstream trypomastigotes isolated from humans. In this report, we show that it has been possible to initiate and maintain infective forms of *T. rhodesiense* in vitro on Chinese hamster (*Cricetulus griseus*) lung cells (ATCC CCL 16 Don). The trypanosomes maintained on these tissue culture cells are infective to mammalian hosts and possess the L-α-glycerophosphate oxidase system characteristic of bloodstream forms.

The Chinese hamster lung cells were obtained from the American Type Culture Collection, Rockville, Maryland. They were maintained in RPMI 1640 (Gibco) supplemented with 25 m*M* Hepes buffer, 20 m*M* sodium bicarbonate, and 20 percent heat-inactivated FBS (KC Biological) and, per 100 ml, penicillin (10,000 U), fungizone (25 μg), streptomycin (10,000 mg), and kanomycin (12.5 μg). The morphology of the tissue cell line was fibroblast-like. An inoculum of 1.8×10^6 viable tissue culture cells multiplied one to three times in 4 days in the described culture medium at 37°C in an atmosphere of 5 percent CO_2 and 95 percent air. The tissue culture cells were used just prior to becoming confluent, usually 3 to 4 days after a flask was inoculated.

The preparation of the trypanosomes from rats was simplified over the procedures described by Hirumi *et al.* (*2*). Successful cultures were established with a pleomorphic strain of *T. rhodesiense* EATRO 1895 from normal or lethally irradiated (800 rads) male rats (weighing 200 g) that were inoculated intraperitoneally with 2×10^6 trypanosomes. When the parasitemia reached a level of 1×10^8 to 5×10^8 trypanosomes per milliliter 3 to 4 days later, the rats were bled by cardiac puncture. From 90 to 95 percent of the trypanosomes present had the morphology of the slender forms, but some stumpy trypanosomes were also observed. The trypanosomes were centrifuged at 1025*g* at 4°C and the buffy coat was removed. Special precautions were taken to prevent the removal of red blood cells below the buffy coat. The trypanosomes were washed twice with sterile Hanks balanced salt solution with 5 percent

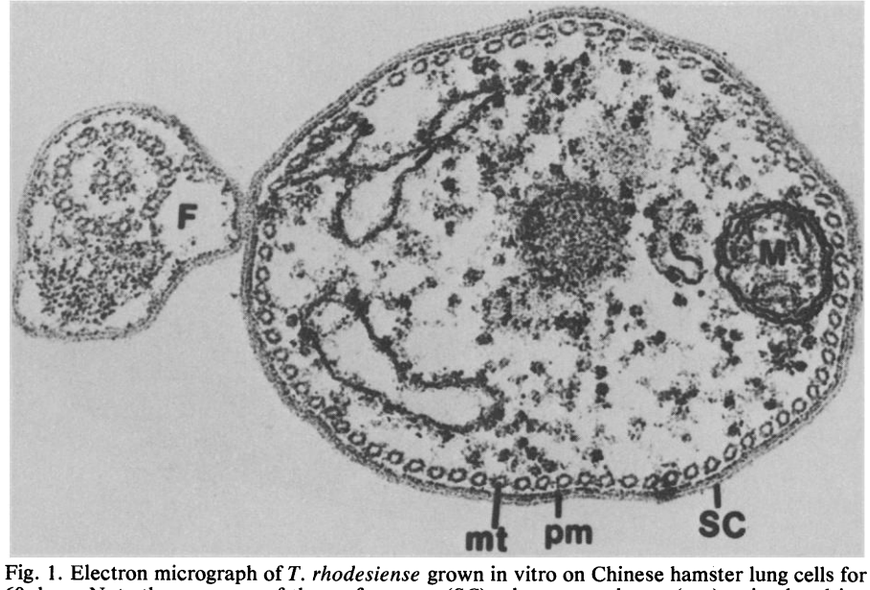


Fig. 1. Electron micrograph of *T. rhodesiense* grown in vitro on Chinese hamster lung cells for 60 days. Note the presence of the surface coat (*SC*), plasma membrane (*pm*), mitochondrion (*M*), and the microtubules (*mt*). The surface coat also surrounds the flagellum (*F*) (×100,000).

0036-8075/78/1117-0763$00.50/0

Table 1. Growth of *T. rhodesiense* on Chinese hamster lung cells.

Day	Mean cell number* (trypanosomes per milliliter)
0	1.0×10^5
1	4.5×10^5
2	3.5×10^6
3	5.4×10^6
4	3.4×10^6
5	3.3×10^6

*One-third of the culture medium was changed daily. The counts presented are those before the culture was changed and each represents three replicate samples per day.

(weight to volume) sodium citrate. The washed trypanosomes were inoculated into a total of 5.0 ml of the culture medium in T-25 Falcon flasks (Fisher Scientific) or into 15.0 ml in T-75 flasks. Most of the successful experiments occurred in the cultures initiated in the T-75 flasks.

A typical experiment was performed by inoculating a T-75 flask containing a monolayer of Chinese hamster lung cells with an initial concentration of 1×10^5 bloodstream trypomastigotes per milliliter. The cultures were incubated at 37°C in an atmosphere of 5 percent CO_2 and 95 percent air in the culture medium. The trypanosome numbers remained low, less than 1×10^5 cells per milliliter for 10 days. The trypanosome population then began to increase and on day 12 reached 5×10^5 cells per milliliter. One-third of the medium was then changed and a new flask started at a concentration of 4×10^5 cells per milliliter. The trypanosome numbers in both flasks then began to increase markedly. By changing one-third of the medium daily, the parasites have been maintained in vitro at a concentration of 3×10^6 to 5×10^6 cells per milliliter over Chinese hamster lung cells for 80 days. In our routine procedures, the tissue culture cells are changed every 7 to 10 days.

Additional trypanosome cultures from the flask containing the original Chinese hamster cells were initiated on other Chinese hamster lung cells. The cultures were initiated at a concentration of 1×10^5 cells per milliliter. The results of a typical experiment on Chinese hamster lung cells are shown in Table 1. On day 3 after inoculation, with one-third of the cell medium changed daily, the cell count was 5.4×10^6 cells per milliliter, reflecting a population doubling time of approximately 9 to 11 hours. The daily trypanosome count continued at 3.0×10^6 to 5.0×10^6 cells per milliliter until they were transferred to a new flask.

On Chinese hamster lung cells, the trypanosomes grow not only in the medium but also in spaces between the tissue culture cells. This occurs particularly after the trypanosomes are established and growing at a concentration above 2×10^6 cells per milliliter. A similar observation was also reported by Hirumi *et al.* (*2*) for *T. brucei* growing on bovine fibroblast-like tissue culture cells.

Giemsa-stained preparations of *T. rhodesiense* maintained in vitro on Chinese hamster lung cells revealed that the kinetoplast is subterminal, clearly distinguishing the trypanosomes as long, slender trypomastigotes. The ultrastructure of *T. rhodesiense* maintained on Chinese hamster lung cells are shown in Figs. 1 and 2. The surface coat is evident as well as the mitochondrion with few cristae, both features characteristic of slender bloodstream trypomastigotes.

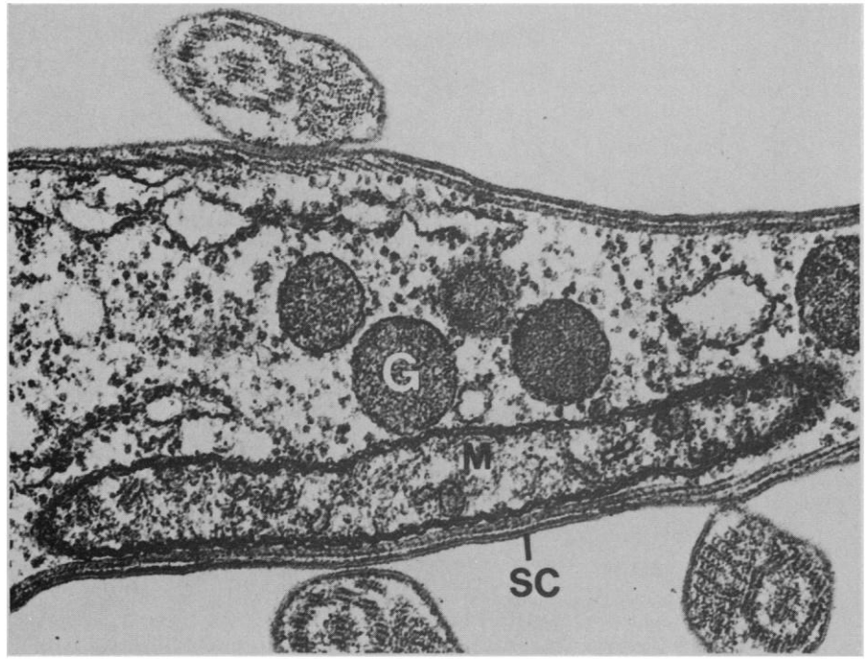
Table 2. Infectivity of *T. rhodesiense* grown on Chinese hamster lung cells.

Trypanosomes	Inoculum of trypanosomes	Days after inoculum before infection developed*
Cultured infective trypanosomes	1×10^6	4
Cultured infective trypanosomes	1×10^5	5
Bloodstream stabilates	1×10^6	4
Bloodstream stabilates	1×10^5	5

*The *T. rhodesiense* trypanosomes had been maintained on Chinese hamster lung cells for 60 days. The infectivity tests are the results of two different experiments with six mice in each experiment.

Experiments to test the infectivity of the cultures were performed with stabilates of bloodstream trypomastigotes of *T. rhodesiense* as controls. The mice were inoculated with 10^5 and 10^6 cells. The results are given in Table 2. Mice or rats inoculated with slender trypomastigotes from bloodstream stabilates or cultured trypomastigotes required the same period before parasitemias developed. In contrast, procyclic trypomastigotes grown in the absence of tissue culture cells were not infective for mice.

To determine the terminal oxidases



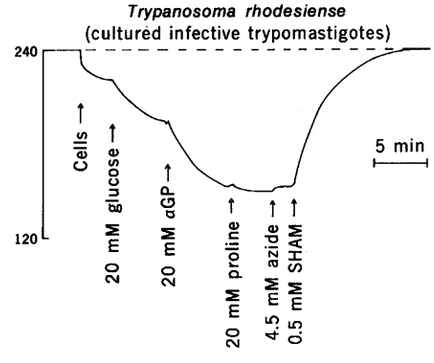


Fig. 2 (left). Electron micrograph of *T. rhodesiense* grown on Chinese hamster lung cells for 60 days. The mitochondrion (*M*) has few cristae. Note the presence of the surface coat (*SC*) and the glycosome (*G*) (×45,000).
Fig. 3 (right). Curve of the steady-state oxygen kinetics of *T. rhodesiense* maintained in vitro for 60 days on Chinese hamster lung tissue culture cells. The trypanosomes were at a concentration of 1.0×10^8 (*α-GP*, α-glycerophosphate oxidase; *SHAM*, salicylhydroxamic acid).

present in the cultured infective forms, we measured the steady-state concentration of the terminal oxidases present. Culture medium (90 ml) from three inoculated Chinese hamster cell cultures was removed at a concentration of 2.5×10^6 trypanosomes per milliliter. The organisms were collected by centrifugation and the steady-state oxygen levels measured (Fig. 3). Salicylhydroxamic acid (SHAM) inhibited 90 percent of the cell respiration. Azide had little effect, inhibiting 7 percent of the cell respiration. Little significant azide- and SHAM-insensitive respiration was observed. These results are similar to our previous results for bloodstream trypomastigotes of *T. brucei* (*4*). They clearly indicate that the SHAM-sensitive α-glycerophosphate oxidase is the predominant terminal oxidase in cultured infective trypomastigotes of *T. rhodesiense*.

The procedures described herein provide a simplified technique for initiating and maintaining large quantities of bloodstream trypomastigotes of *T. rhodesiense* in culture on an established tissue culture cell line. The cultured infective trypomastigotes have also been stored as frozen stabilates at −90°C (that is, culture mixed 1 : 1 with 20 percent glycerol in the culture medium). The thawed stabilates remained infective for mice and rats and continued to develop in vitro on Chinese hamster lung cells.

At present, the *T. rhodesiense* trypomastigotes have retained infectivity after being maintained for 80 days in vitro at 37°C. The concentration of trypanosomes obtained in our experiments (3×10^6 to 5×10^6 cells per milliliter) should provide adequate numbers of cells to allow further biochemical and immunological studies, and this system may prove valuable in studies of the process of trypanosome antigenic variation.

GEORGE C. HILL
SUSAN PETERLIN SHIMER
BYRON CAUGHEY
L. SCOTT SAUER

Department of Pathology, Graduate Program in Cell and Molecular Biology, Colorado State University, Fort Collins 80523

References and Notes

1. The causative agents of African trypanosomiasis are bloodstream trypanosomes. The proper term for these protozoa is bloodstream trypomastigotes.
2. H. Hirumi, J. J. Doyle, K. Hirumi, *Science* **196**, 992 (1977); *Bull. WHO* **55**, 405 (1977).
3. The abbreviations Hepes and RPMI are for 4-(2-hydroxyethyl)-1-piperazineethanesulfanic acid and Roswell Park Memorial Institute, respectively.
4. G. C. Hill, in *Biochemistry of Parasites and Host-Parasite Relationships*, H. Van den Bossche, Ed. (Elsevier/North-Holland, Amsterdam, 1976), pp. 31–50.
5. Supported by an NIH research career development award (1-KO-4, AI-70813-04) to G.C.H., an NIH research grant (1-AI-11622-04) and a contract from the U.S. Army Medical Research and Development Command (DAMD-17-74-C-4046). We thank J. Sugimoto and R. Medville for help in preparing the electron micrographs and S. K. Swets for help in typing the manuscript.

8 June 1978; revised 1 August 1978

Cellular Interactions Uncouple β-Adrenergic Receptors from Adenylate Cyclase

Abstract. *C6 glioma cells and B104 neuroblastoma cells both possess adenylate cyclase activity, but only C6 cells have β-adrenergic receptors. However, when cocultured with B104 cells, C6 cells show a marked decrease in their ability to accumulate adenosine 3′,5′-monophosphate upon stimulation with β receptor agonists. Since both β receptors and cholera toxin–stimulated adenylate cyclase activities are present in C6/B104 cocultures, we conclude that the β receptor/adenylate cyclase transduction mechanism in cocultured C6 cells is uncoupled.*

The means by which cells communicate with each other include syncitial events [that is, the passage of ions or small molecules through intercellular junctions (*1*)], events involving soluble factors (for example, hormones, neurotransmitters), and those utilizing membrane-associated elements in intimate cellular contact. This last mode of communication is believed to involve cell surface molecules on an "instructor" cell interacting with its counterparts on a target cell to effect specific changes in the target cell's physiology (*2*). The membrane-associated molecules thought to mediate these interactions include gangliosides (*3*), glycosaminoglycans (*4*), and glycoproteins (*5*). Although the effects of neurohormonal communication have been well characterized in a variety of systems (*6*), similar biochemical effects of either syncytial- or surface molecular-interactions between cells of different types have been studied to a lesser extent (*7*).

In this report, we describe a technique for coculturing two cell types for studies of these intimate types of cellular interactions. This technique offers the advantages of enabling one to monitor the absolute number of each cell type at any time, as well as to coculture cells with very different growth rates without one cell type overgrowing the other. Using C6 glioma and B104 neuroblastoma cells—both cloned cell lines from chemically induced tumors of the rat central nervous system (*8, 9*)—we found that intimate cellular interactions with B104 cells cause the β-adrenergic receptors on C6 cells to become uncoupled from adenylate cyclase.

Trypsinized C6 (subclone 2B) cells, some of the cells previously labeled with [^{3}H]thymidine, were plated onto either confluent beds of B104 cells (C6/B104 cocultures) or onto tissue culture plastic surfaces (C6-only cultures) (*10*). The density of C6 cells in the inoculum was such that only two to three rounds of cell division could occur before their growth became contact-inhibited. (Confluent cultures of B104 cells not receiving C6 cells are termed "B104 only.") These cultures were then allowed to grow for various periods of time. Similar cultures were fixed and processed for autoradiography (*11*). To estimate the numbers of each cell type growing in coculture, we first examined autoradiographs of C6-only cultures and determined the proportion of C6 cells containing label. This ratio, divided into the number of labeled cells seen in autoradiographs of C6/B104 cocultures, yields the total number of C6 cells growing on the B104 cell bed. (At least three rounds of cell division can occur before the amount of label per daughter cell falls below that detectable by autoradiography.) The number of B104 cells growing in cocultures was obtained by subtracting the amount of C6 DNA (*12*) from total coculture DNA and then by dividing this remainder by the amount of DNA per B104 cell.

Figure 1 shows that from day 3 after the addition of C6 cells, there were marked differences among the cultures in their abilities to accumulate adenosine 3′, 5′-monophosphate (cyclic AMP) in response to incubation with the potent β-adrenergic agonist isoproterenol. The C6-only cultures showed a steady increase in this β responsiveness as a function of time. In contrast, C6 cells in the C6/B104 cocultures maintained a low level of β responsiveness, even after the C6 cells had reached their maximum cell density (inset, Fig. 1). The B104-only cultures showed a consistent, yet statistically insignificant (*13*) increase in cyclic AMP throughout the experimental period (for example, see Table 1). Similar

0036-8075/78/1117-0765$00.50/0