CULTIVATION OF *TRYPANOSOMA CRUZI* USING HUMAN DIPLOID CELLS

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Abstract: Interaction between normal diploid cells which had been established from human lung tissue and *Trypanosoma cruzi* (T. c) stock newly isolated from an Ecuadorian patient with Chagas' disease was studied. Normal diploid cells were found to have susceptibility to invasion by T. c and capacity to support the parasite growth. Intracellular parasites multiplied with a doubling time of 16.9 hours, and after 4 days of infection, trypomastigotes were released into culture supernatant. Development of T. c was relatively synchronized in the infection with parasite: host cell ratio of 20:1, providing broad form trypomastigotes with purity of more than 95 per cent in culture supernatant on day 4. Amastigotes were observed during the later stage of cultivation, resulting from degeneration of host cells. Two morphologically distinct forms of trypomastigotes, broad and slender, were obtained. Broad form predominated at early stage of cultivation and then proportion of slender form gradually increased.

INTRODUCTION

A wide range of host cell systems including primary cell cultures and permanent cell lines has been reported to support growth of *Trypanosoma cruzi* (T. c), the causative agent of Chagas' disease (Brener, 1973). Such growth, however, varies depending upon host cell types and parasite strains, influencing multiplication and transformation of the parasite in host cells (Dvorak and Howe, 1976; Bertelli and Brener, 1980). Until now, the techniques for a large scale and standardized in vitro production of trypomastigote forms free from contamination with other forms have been exploited. A culture system using rat muscle cells irradiated for stopping host cell division (Schmatz and Murray, 1982), and a combination of murine muscle-derived cell line with cloned T. c in continuous culture system (Hudson *et al.*, 1984) have been established for production of large homogenous populations of trypomastigotes. Sander-son *et al.* (1980) succeeded in obtaining trypomastigotes with a very low level of contamination with other forms by employing human diploid cells MRC-5 as host cells. In the present study, we employed normal diploid cells which had been established from the human fetal lung tissue in our laboratory, in an attempt to assess their susceptibility to invasion by T. c and capacity to support complete differentiation of amastigotes to trypomastigotes. In addition, we attempted to establish an Ecuadorian stock of T. c, since little is known about the characterization of T. c strains or stocks from Ecuador.

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MATERIALS AND METHODS

Parasite: Parasites of T.c used in the present study were newly isolated from an Ecuadorian patient with Chagas' disease in 1982. The patient, 10-year-old male, resided at Balzal, Guayaquil city, where the infection with T. c is sporadically endemic. He was pointed out the signs that appeared to be Chagas' disease at a local hospital, and consulted INHMT (Instituto Nacional Higiene y Medicina Tropical, Guayaquil) to confirm diagnosis. Examination of the fresh blood and blood smear, and xenodiagnosis using *Triatoma dimidiata* were positive for T. c. The parasites obtained from the peripheral blood were maintained in Novy, MacNeal, and Nicoll's (NNN) medium by serial passage. The stock of T. c was referred to as "Guayas E".

Normal diploid cell: Normal diploid cells with a finite life, HAIN-55, were used as host cells. They were fibroblastic cells established from human fetal lung tissues in our laboratory in 1973 and have been maintained since then (Okumura *et al.*, 1979). HAIN-55 were grown in Basal Medium Eagle (BME) supplemented with 10 per cent fetal calf serum (FCS) and were incubated at 37°C in air with 5 per cent CO₂ in a tissue culture plastic dish. They showed stable non-dividing monolayers at confluence. Before transfer, the cells were dislodged from plastic dish after a brief treatment with 0.25 per cent trypsin and then seeded into a new dish.

Parasite infection: Initially, normal diploid cells were infected by addition of T. c grown in NNN medium, and trypomastigotes obtained from the first passage were used in subsequent experiments in order to minimize the biological alteration which may occur during continuous passages. Host cells (4×10^5 in 2 ml of BME with 10% FCS) were seeded into a tissue culture dish (35 mm in diameter) in which coverslip was set, and were incubated at 37°C in air with 5 per cent CO₂ for 24 hours to allow attachment on the dish. Infection was initiated by adding trypomastigotes at various parasites: host cell ratios ranging from 1:1 to 20:1. Five hours later, T. c remained in culture supernatant were removed by repeated washing with the medium, then continued the cultivation.

Morphological observations: The progress of infection was monitored using a inverted microscope with phase-contrast optics. At various periods of time after infection, each coverslip was removed, washed in phosphate-buffer saline, fixed with methanol and stained with Giemsa. The percentage of host cells infected with T. c and the average numbers of amastigotes per infected cell were determined by light microscopic examination. The number of parasites released into culture supernatant was counted in a haemocytometer. The morphology of parasites was assessed by examination of Giemsa-stained preparations.

RESULTS

Growth of parasites in normal diploid cells: Susceptibility of normal diploid cells, HAIN-55, to infection with Guayas E stock of T. c was examined by adding various concentrations of parasites, and percentage of infected cells with T. c was determined at 24 hours after inoculation. Percentages of infected cells in these culture system depended on levels of inoculum and more than 80 per cent of infected cells were obtained when infected with parasite: host cell ratio of 20:1 (Tabel 1). The development of intracellular stage of parasites is shown in Figure 1. The intracellular parasites multiplied exponentially within host cells and

Table 1 Susceptibility of normal diploid cells (HAIN-55) to invasion by T.c at various parasite : host cell ratios

Ratio (parasite/cell)	Infected cells (%) ^{a)}
20 : 1	80.9 ± 4.5 ^{b)}
10 : 1	38.3 ± 0.8
5 : 1	15.7 ± 3.3
2.5 : 1	11.4 ± 2.1
1 : 1	7.6 ± 1.5

^{a)} Determined from the number of infected cells/500 counted.

^{b)} Mean ± S.D. of at least three experiments.

Figure 1 Growth of intracellular parasite of T. c in normal diploid cells, HAIN-55.

the average number of parasite per infected cell reached 43.5 on day 4 after inoculation wherein transformation into trypomastigotes were commonly observed in host cells loading a large number of intracellular parasites. Doubling time was estimated to be 16.9 hours from growth curve during log-phase multiplication of the trypanosoma population.

Parasite yield: Figure 2 shows number of trypomastigotes released from host cells into culture supernatant. The time necessary to yield peak production and to persist production of trypomastigotes was closely related to levels of inoculum; in the infection with parasite : host cell ratio of 20 : 1, number of trypomastigotes yielded reached a maximum population of 2.4×10^7 on day 5, and then decreased rapidly. The 5 : 1 infection gave trypomastigote production

Figure 2 Production of trypomastigote forms at various levels of inoculum. Each point represents the number of trypomastigotes released into culture supernatant in the infection with parasite : host cell ratio of 20 : 1 (▲), 5 : 1 (◆), and 1 : 1 (●).

from day 3 to 16, displaying biphasic pattern of parasite development: First peak from day 5 to 6 and second peak from day 12 to 14. The longest trypomastigote production was obtained in the infection with parasite: host cell ratio of 1:1, in which release of trypomastigotes persisted until day 24. However, infections with 1, 5 and 20 parasites/host cell gave similar overall yields: The number of parasites produced were 4.3 , 3.9 and 4.0×10^7 per dish, respectively. Amastigotes resulted from degeneration of host cells were identified in culture supernatant later than appearance of trypomastigotes (Figure 3). The time of appearance of amastigotes varied in relation to levels of inoculum, and a minimum contamination with amastigotes was obtained when host cells were infected with parasites: host cell ratio of 20:1. The overall productions of amastigotes in the infections with 1, 5 and 20 parasites/host cell were 1.4 , 2.0 and 0.6×10^7 per dish, respectively.

Polymorphism of produced trypomastigote: Two forms of trypomastigote were distinguished after staining preparation with Giemsa. The broad form has the characteristic "C" shape with a terminal kinetoplast, an undulating membrane and a flagellum. The slender form

Figure 3 Appearance of amastigote form at various levels of inoculum. Each point represents the number of amastigotes released into culture supernatant in the infection with parasite: host cell ratio of 20:1 ($\blacktriangle$), 5:1 ($\blacklozenge$), and 1:1 ($\bullet$).

Figure 4 Proportion of slender form in total trypomastigotes produced. Each point represents the percentage of slender form in total trypomastigotes released into culture supernatant in the infection with parasite: host cell ratio of 20:1 (A), 5:1 (B), and 1:1 (C).

is narrower and longer, with a sub-terminal kinetoplast and no obvious undulating membrane. Figure 4 shows the percentage of slender form in total trypomastigotes. Broad form predominated in the early stage of cultivation and thereafter proportion of slender form gradually increased to around 40 per cent. These patterns were observed in all levels of inoculum employed in the present study.

DISCUSSION

An employment of normal human diploid cells for the production of materials for use in human was recommended by Jacobs *et al.* (1970). In order to develop a standardized culture system for *T. c.*, we also employed normal diploid cells which had been established from human fetal lung tissues in our laboratory.

The results demonstrated that normal diploid cells, HAIN-55, were susceptible to invasion by Guayas E stock of *T. c.* and capable of supporting parasite growth. Amastigotes of *T. c.* were multiplied within the host cells with a doubling time of 16.9 hours, and after 4 days of infection, bloodstream form, trypomastigotes, were released into culture supernatant. A relatively synchronized development of *T. c.* was provided in the infection with high ratio of parasite: host cell (20:1). More than 80 per cent of host cells were infected and 10^7 trypomastigotes per dish were produced on day 5 of infection. Amastigotes appeared in culture supernatant possibly as a result of host cell rupture before complete transformation of *T. c.* when the monolayer became fragile or detached. The condition of host cells appears, therefore, to be an important factor for the purpose of obtaining homogenous trypomastigotes.

Two morphologically distinct forms of trypomastigote, broad and slender forms, were obtained from culture supernatant. Sanderson *et al.* (1980) reported that the broad form predominated in early tissue-culture passage using human diploid cells, but after about 10 passages the slender form predominated. In the case of HAIN-55 and Guayas E of *T. c.* combination, slender form predomination was not noted after multiple tissue-culture passages, although the proportion of slender form increased to around 40 per cent at the later stage of cultivation. Brener (1965, 1968), in a study on polymorphism of *T. c.* in experimentally infected mice, has observed the correlation between polymorphism and the course of parasitemia, the mortality rate and susceptibility to host's immune response. However further biological significances of the polymorphism are unclear.

It has been well documented that the prevalence and severity of Chagas' disease are confined to certain geographic areas may be associated with different strains of parasites (Miles, 1983). The characterization of *T. c.* stocks from various regions of South America and the relationship between geographical distribution and pathology of Chagas' disease in man has been reported recently (Miles *et al.*, 1981; Tibayrens and Miles, 1983; Miles *et al.*, 1984; Widmer *et al.*, 1985). Further studies for clarifying the characteristics of Guayas E stock are presently undertaken.

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ヒト二倍体細胞株を用いた *Trypanosoma cruzi* の培養

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エクアドル国産の Guayas E 株 *Trypanosoma cruzi* (T. c) のヒト正常二倍体細胞への感染と、その増殖を試みた。実験に用いた T. c は、エクアドル国グアヤキル市在住の急性シャーガス病患者より分離し、Guayas E 株と命名した。宿主細胞として用いた細胞株は、ヒト胎児肺組織由来の二倍体線維芽細胞である。35 mm シャーレに 4×10^5 宿主細胞を植え込み、虫体：宿主細胞を 1:1 から 20:1 の割合で感染し、24時間後に感染率を観察すると 20:1 では、感染率は80%以上であった。宿主細胞内での T. c 増殖は、指数関数的増加曲線を示し、感染後4日目の細胞内虫体数は 43.5 で doubling time は、16.9時間と推測された。35 mm シャーレを用い、虫体：宿主細胞を 1:1, 5:1, 20:1 で感染すると、遊出した全 trypomastigote は、それぞれ $4.3, 3.9, 4.0 \times 10^7$ で大差はみられないが、遊出全 amastigote は、それぞれ $1.4, 2.0, 0.6 \times 10^7$ であり、20:1 比率での感染早期に amastigote の混入が少なく、多量の trypomastigote が回収できる。遊出した trypomastigote には slender 型 T. c と broad 型 T. c の両型が確認され、培養早期には、90%以上が broad 型であるが、培養を持続すると slender 型の遊出割合が増加する傾向にあった。

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