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Quantitative Determination of Growth of Amastigotes and Trypomastigotes in an In Vitro Cultivation System of HeLa Cells Infected with *Trypanosoma cruzi*

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ABSTRACT To determine the growth and morphogenesis of *Trypanosoma cruzi* in mammalian host cells, we have examined the conditions for in vitro propagation of amastigotes and trypomastigotes of *T. cruzi* in HeLa cells. Within one day after the infection, the developmental stage of parasites changed from the trypomastigote form to amastigote form in the host cells. The rate of infection by *T. cruzi* of the host cells increased in an inoculum- and time-dependent manner. The amastigotes proliferated rapidly by binary fission, reaching a peak of the average number of about 20 amastigotes per infected HeLa cell on day 6 after infection. Simultaneously, the alteration of morphology from amastigotes to trypomastigotes also took place within the host cells, the latter disrupting the host cells and again appearing in the culture medium. On day 8, the number of amastigotes decreased and that of trypomastigotes increased, inside and outside of HeLa cells, respectively. The system established in this study may facilitate 1) the quantitative propagation of amastigotes and trypomastigotes and 2) the study of molecular and cellular mechanisms that underlie stage-specific transformation of *T. cruzi*.

INTRODUCTION

Trypanosoma cruzi, the etiologic agent of Chagas' disease in man in Central and South America, exhibits three major developmental stages during its life cycle in the insect vector and in various mammalian hosts (Brener 1973, de Souza 1984). In the insect vector, this protozoan parasite occurs as the epimastigote form, while it shows two forms, trypomastigote and amastigote, in mammals. The trypomastigote form, which has a flagellum for locomotion, circulates in the bloodstream and is believed to be in the nondividing stage. On the other hand, the

amastigote form, which has no free flagellum, parasitizes the cytoplasm of host cells and proliferates intracellularly. Little is known about the molecular and cellular mechanisms involved in morphological changes from one stage to the other and the interactions that must exist at the interface between the parasites and host cells. To understand and analyze the biological and biochemical differences between these developmental stages of *T. cruzi* and mammalian host cells, we have attempted to construct an in vitro culture of host cells infected with *T. cruzi*.

Several research groups reported the in vitro infection of various types of host cells by *T. cruzi* trypomastigotes (Dvorak and Howe 1976, Bertelli and Brener 1980, Schenkman et al. 1988). Although *T. cruzi* prefers to invade muscle cells, particularly heart muscle cells in vivo, the parasite is also capable of infecting different host cell types in vitro. Since the mammalian cell lines adapted to liquid culture are more advantageous than primary culture cells to yield the reproducible growth under defined conditions, we have used HeLa cells as the host for in vitro infection by *T. cruzi* in this study. Dvorak and Crane (1981) also used HeLa cells for the analysis of the effect of host cell cycle on the *T. cruzi* infection. The purpose of our study was, however, aimed to quantitatively determine the time course of amastigote growth and of morphological changes from amastigotes to trypomastigotes inside host cells.

MATERIALS AND METHODS

Materials

The human cancer cell line, HeLa, and the infection complex of the HeLa cells (Kaneda et al. 1986) and *Trypanosoma cruzi*, Tulahuene strain (Taliaferro & Pizzi 1955), were successively subcultured every 3 to 4 days at cell densities of 3-5 x 10^5 cells per ml in 25 cm^2 plastic flasks (Corning-Iwaki Glass, Tokyo). Eagle's minimum essential medium (MEM) was from Nissui Seiyaku Co., Ltd., Tokyo, and fetal bovine serum from Daiichi Pure Chemicals Co., Ltd., Tokyo. Diff-Quik was purchased from Kokusai Seiyaku Co., Ltd., Tokyo. Other chemicals were commercial products of the highest grade.

Subculture

At the late exponential phase of growth, the trypsin-treated HeLa cells were subcultured every 3 to 4 days in MEM (5 ml) supplemented with heat-inactivated (56°C, 30 min) 10% fetal bovine serum in 25 cm^2 flasks. The host-parasite infection complex, i.e., the HeLa cells infected with *T. cruzi*, was also cultivated in the same manner. These subcultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

In vitro infection

Exponentially growing HeLa cells were harvested from the subcultures and inoculated into 24-well plates (Corning-Iwaki Glass) with an inoculum size of 5 x 10^3 cells per ml per well, followed by incubation for 2 days at 37°C in 5% CO_2 in air. The HeLa cells were then infected with *T. cruzi* trypomastigotes (1 x 10^4, 1 x 10^5 or 1 x 10^6 parasites per well). Trypomastigotes were obtained as follows. The medium (5 ml) of the preceding subcultures of host-parasite infection complexes was transferred into 15-ml centrifuge tubes and the infected cells attached to the surface of the flask were washed with 5 ml of phosphate-buffered saline (PBS). The combined medium and wash containing trypomastigotes was centrifuged at 1000 x *g* for 3 min at room temperature to remove the contaminated host cells and their debris. Trypomastigotes were then collected by centrifugation of the supernatant at 1600 x *g* for 10 min, suspended in 100 µl of MEM, and counted on a Neubauer haemocytometer using a light microscope. After diluting with an appropriate amount of MEM, 10 µl of parasite suspension was added to each well containing precultured HeLa cells.

Determination of the rate of infection and the number of parasites

On days 2, 3, 4, 6, and 8 after infection, the medium in each well was withdrawn to determine the number of trypomastigotes, as described above. The HeLa cells remaining at the bottom of the well were fixed and stained with Diff-Quik for observation using an inverted light microscope. The percentage of infected host cells that contained more than one amastigote, and the mean number of amastigotes per infected cell were determined by analyzing more than 200 HeLa cells distributed in randomly chosen microscopic fields.

RESULTS AND DISCUSSION

Changes in the rate of infection

As shown in Fig. 1, the rate of infection by *T. cruzi* of HeLa cells increased constantly in an inoculum-size-dependent manner and, on day 8 after infection, reached 82 and 51% in the cases of the inoculation with 1 x 10^5 and 1 x 10^4 trypomastigotes, respectively. Infection with 1 x 10^6 trypomastigotes caused much damage to the host HeLa cells by day 4, and this inoculum was thus thought to be inadequate for the time-course observation of *T. cruzi* infection to HeLa cells under the conditions used.

Changes in the number of amastigotes per infected HeLa cell

Figure 2 shows the time course of changes in the average number of amastigotes per infected HeLa cell. The number of amastigotes reached a peak of about 20 in an infected host cell on day 6, after which a decrease was observed. These results strongly suggest that the amastigotes

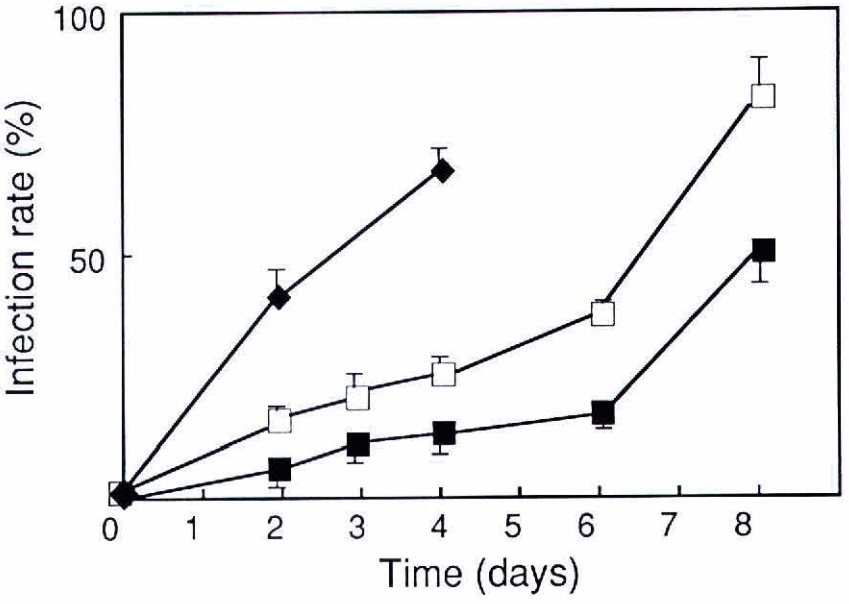

Fig. 1 Changes in the rate of infection of HeLa cells. HeLa cells (5×10^3) were precultured for 2 days as described in the Materials and Methods, and on day 0, different numbers of *T. cruzi* trypomastigotes were inoculated. ■, 1×10^4 parasites; □, 1×10^5 parasites; ◆, 1×10^6 parasites. The values are the means of triplicates, the bars representing SD.

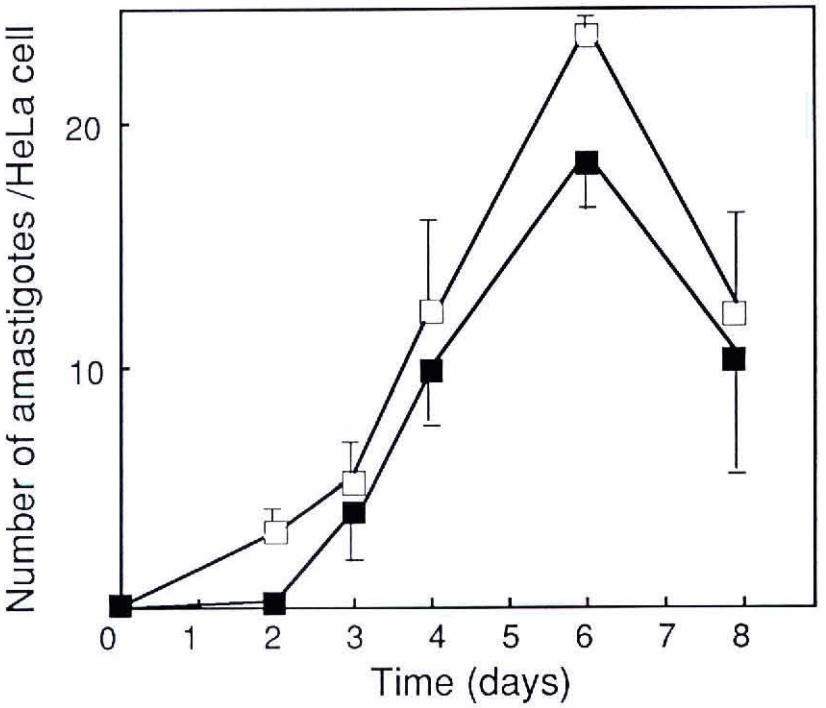

Fig. 2 Changes in the number of *T. cruzi* amastigotes per infected HeLa cell. Trypomastigotes (■, 1×10^4 and □, 1×10^5 cells) were inoculated as described in Fig. 1. The values are the means of triplicates, the bars representing SD.

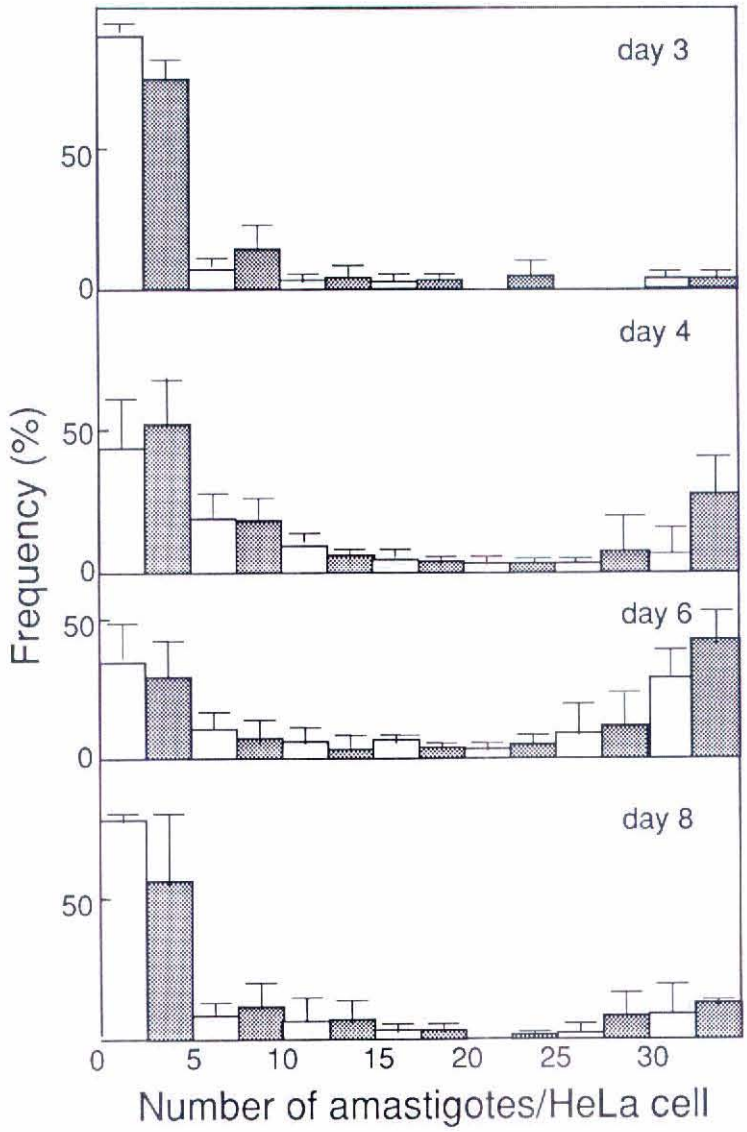

Fig. 3 Changes in frequencies of the number of *T. cruzi* amastigotes per infected HeLa cell. Trypomastigotes (▩, 1 x 10^4 and □, 1 x 10^5 cells) were inoculated as described in Fig. 1, and frequencies of the number of amastigote in the individual infected host cells were tabulated. The values are the means of triplicates, the bars representing SD.

proliferate rapidly by binary fission in the host cells until day 6, and that the parasites then transform into trypomastigotes, resulting in the breakdown of host cells and the decrease of amastigotes in number per infected HeLa cell. Although inoculum sizes of trypomastigotes differing by 10-fold were applied, only about 1.2-fold difference in the maximum number of amastigotes was obtained on day 6 (Fig. 2).

Figure 3 shows frequencies of the number of amastigotes per infected HeLa cell. More than 80% of the infected HeLa cells contained 1 to 5 amastigotes on day 3. HeLa cells containing more than 20 amastigotes may indicate that more than one trypomastigotes is capable of invading the same host cell, although the doubling time of amastigotes in the host HeLa cells was not determined in this study. On day 6, the frequencies of the infected host cells containing 1 to 5 amastigotes decreased to 30-40%, whereas those of infected cells that contained more than 25 amastigotes were increased. The frequency pattern on day 8 resembled the pattern obtained on day 3. The increased frequency of HeLa cells with 1 to 5 amastigotes on day 8 is probably indicative of the secondarily infection of non-infected host cells by trypomastigotes raised in the initially infected host cells.

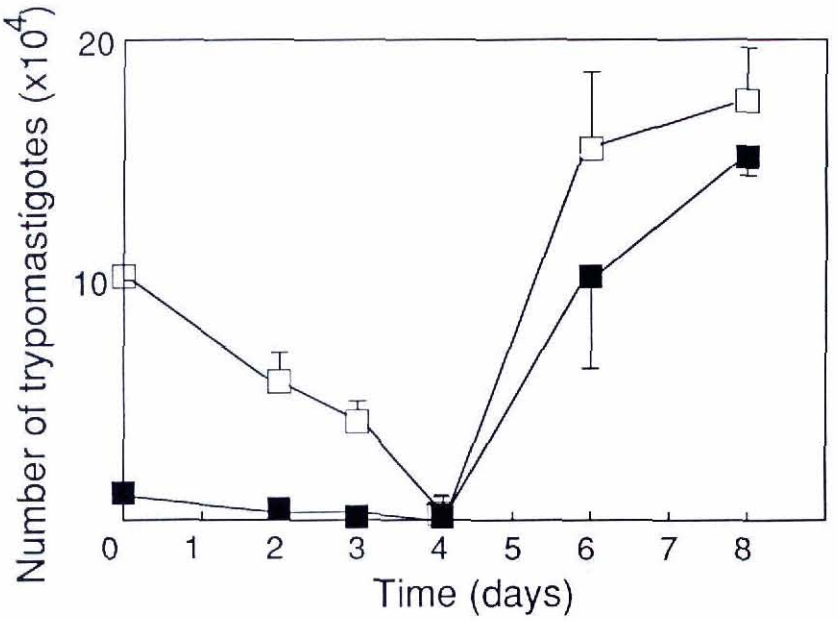

Fig. 4 Changes in the number of trypomastigotes in culture medium. *Trypanosoma cruzi* trypomastigotes (■ , 1 x 10^4 and □ , 1 x 10^5 cells per well) were inoculated on day 0, and the number of trypomastigotes in the medium was determined as described in the Materials and Methods. The values are the means of triplicates, the bars representing SD.

Changes in the number of trypomastigotes in culture medium

When 1 x 10^5 trypomastigotes were inoculated, a large number of parasites still remained in the culture medium on days 2 and 3 (Fig. 4) and were spontaneously transformed into extracellular amastigotes (data not shown); in a separate preliminary experiment, we confirmed that these amastigotes neither infected the host cells nor transformed into trypomastigotes. On the other hand, the infection with 1 x 10^4 trypomastigotes did not yield parasites remaining in the medium on days 2, 3 and 4 (Fig. 4). On day 6, trypomastigotes appeared again in the culture medium and, on day 8, freshly appearing 1.7 x 10^5 trypomastigotes (about 17 times) were recovered.

The results obtained in this study are consistent with the following observations. HeLa cells (5 x 10^3 cells) were incubated for 2 days and then infected with *T. cruzi* trypomastigotes (1 x 10^4 parasites). Within 1 day after the infection, trypomastigotes invaded HeLa cells and changed morphologically to amastigotes. On days 2, 3, 4, 6, and 8, the rate of infection of host cells increased to 6.6, 11.6, 14.3, 16.9, and 51.3 %, and the average number of amastigotes per infected cell also increased to 1.0, 3.3, 10.2, 18.6, and 10.6, indicating the rapid growth of parasites up to day 6 after infection. The number of trypomastigotes in the medium were 1.0 x 10^5 and 1.7 x 10^5 on days 6 and 8, respectively. These results indicate that, around day 6, the alteration of morphology from amastigotes to trypomastigotes took place, the latter disrupting the host cells and again appearing in the culture medium.

We have confirmed that the results summarized above are reproducible within a range of experimental errors in our in vitro culture system of the host and parasite cells. This system includes the following steps: 1) the pre-cultivation of host HeLa cells (5 x 10^3 / ml / well) in 24-well plates for 2 days at 37°C in 5%CO_2 in air; 2) the infection of these host cells by *T. cruzi* trypomastigotes (1 x 10^4 ~ 10^5 / well); 3) the determinations of the rate of infection of host cells and of the mean number of amastigotes / infected HeLa cell, and 4) the measurement of the number of trypomastigotes in the medium after infection. It can be expected that our system brings high yields of amastigotes on day 6 and of trypomastigotes on day 8 after infection. In addition, the observation of the transformation from amastigotes to trypomastigotes must be done around day 6 after the infection. Consequently, we have succeeded in establishing a culture system of host cells infected with *T. cruzi* and in determining quantitatively the time course of growth of amastigotes and trypomastigotes. The system established in this study may facilitate 1) the quantitative propagation of amastigotes and trypomastigotes and 2) the study of molecular and cellular mechanisms that underlie stage-specific transformation of *T. cruzi*.

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