Enucleated L929 Cells Support Invasion, Differentiation, and Multiplication of *Trypanosoma cruzi* Parasites

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Cell infection with *Trypanosoma cruzi*, the agent of Chagas’ disease, begins with the uptake of infective trypomastigotes within phagosomes and their release into the cytosol, where they transform into replicating amastigotes; the latter, in turn, differentiate into cytolytically released and infective trypomastigotes. We ask here if the *T. cruzi* infection program can develop in enucleated host cells. Monolayers of L929 cells, enucleated by centrifugation in the presence of cytochalasin B and kept at 34°C to extend the survival of cytoplasts, were infected with parasites of the CL strain. Percent infection, morphology, stage-specific markers, and numbers of parasites per cell were evaluated in nucleated and enucleated cells, both of which were present in the same preparations. Parasite uptake, differentiation and multiplication of amastigotes, development of epimastigote- and trypomastigote-like forms, and initial cytolytic release of parasites were all documented for cytoplasts and nucleated cells. Although the doubling times were similar, parasite loads at 48 and 72 h were significantly lower in the cytoplasts than in nucleated cells. Similar results were obtained with the highly virulent strain Y as well as with strains CL-14 and G, which exhibit low virulence for mice. Cytoplasts could also be infected with the CL strain 24 or 48 h after enucleation. Thus, infection of cells by *T. cruzi* can take place in enucleated host cells, i.e., in the absence of modulation of chromosomal and nucleolar gene transcription and of RNA modification and processing in the nucleus.

Paradigmatic intracellular bacteria inject host cells with plasmid- or chromosome-encoded virulence factors that hijack or sabotage cell functions required for initiation and/or development of the infection (14, 30). The tight control of infection by these organisms may account for the commonly held, although rarely tested, belief that modulation of host cell transcription may not be required in the course of infection with nonviral bacterial pathogens. Less information is available on the infection of cells with unicellular eukaryotic parasites. However, although they are not known to assemble molecular syringes and needles, these pathogens likewise express and/or secrete effector molecules that control cell entry and intracellular targeting or modulate host cell functions required for survival, multiplication, and dissemination in the host (7, 11, 14, 25, 41).

It was also shown that depending on the cell type and functional condition, host cell transcription can be broadly modulated, directly and/or indirectly, by prokaryotic or eukaryotic pathogens, their molecular components, and/or secreted products (14, 21, 22, 35). Some of these responses, common to viral and nonviral pathogens, were linked to stress and to conserved host defense mechanisms, whereas others were pathogen specific (16, 21, 22). It may not always be possible, however, to distinguish responses involved in the protection of host cells or the host from others that favor the pathogen or that may be neutral. Furthermore, it is not known if any of the host transcriptional responses are necessary for completion of the infection.

In the present study, we ask if enucleated L929 fibroblast-like cells can be infected with the kinetoplastid flagellate *Trypanosoma cruzi*, the agent of Chagas’ disease, a zoonosis and anthroponosis carried by hemipteran vectors and widely distributed in Central and South America (2). Of particular interest here are the unorthodox molecular and metabolic peculiarities that *T. cruzi* shares with other kinetoplastids (43) and its elaborate and relatively protracted intracellular life cycle (2, 6, 26, 48). Furthermore, parasite-expressed and -secreted proteases, *trans*-sialidases, and other effectors have been shown to modulate in vitro and in vivo infection with *T. cruzi* (e.g., see references 2, 36, and 37).

Host cell monolayers were enucleated by centrifugation in the presence of cytochalasin B, a procedure that interrupts ongoing chromosomal and nucleolar gene transcription and nuclear RNA processing, thus disabling important cell nucleus-dependent signaling cascades (18). In principle, the infection of cytoplasts by a given pathogen may be unaffected, decreased, or increased in comparison with that of nucleated controls. Furthermore, if nucleated cells express transcription-dependent microbicidal or microbistatic mechanisms (16), infection may be greater in enucleated than in nucleated host cells. In addition, if nonrenewable cytoplasmic factors are required for *T. cruzi* infection, the latter should fall with time after enucleation.

We report here that L929 cytoplasts maintained at 34°C
hosted the nearly complete in vitro intracellular cycle of the virulent strain CL of *T. cruzi*, up to the production of trypomastigotes. These results were extended to strains Y (pathogenic), CL-14, and G (both of low pathogenicity). However, for all of these strains, although overall parasite doubling times were similar in cytoplasts and nucleated cells, parasite loads at 48 and 72 h of infection were significantly smaller in cytoplasts than in their nucleated controls.

**MATERIALS AND METHODS**

Cells, media, and growth conditions. NCTC clone L929 fibrosoarcoma cells, originally derived in 1948 from a male C3H-An mouse, were used in this study. Cells were grown in complete Dulbecco’s modified Eagle’s medium (cDMEM) with 5% fetal bovine serum, 2 g/liter bicarbonate, and 15 mM HEPES. Confluent cultures were trypsinized, and 1 × 10⁷ cells were seeded for 24 h in 2-cm² wells, each containing an 8- by 12-mm “minislide” cut from a standard microscope slide (54). Vero cells (African green monkey kidney fibroblasts) and CHO-K1 cells (Chinese hamster ovary cell line) were grown at 37°C in RPMI 1640 medium supplemented with 5% fetal bovine serum, streptomycin (100 µg/mL), and penicillin (100 U/mL). These cells were used to maintain the in vitro *T. cruzi* cycle.

Parasites, media, and growth conditions. Four *T. cruzi* strains were used in this study. Strain CL was originally isolated from *Triatoma infestans* found in dwellings where people were infected (4); metacyclic trypomastigotes of this strain efficiently infect mice and invade mammalian cells in vitro. CL-14, a temperature-sensitive derivative of the CL strain, is unable to produce patent infection, even when injected into newborn mice, and could not be found upon extensive histopathological analysis of tissues and organs of mice after intraperitoneal or intravenous injection of metacyclic forms (24). Strain G was isolated from an opossum in the Brazilian Amazon. Metacyclic forms of this strain invade mammalian cells in vitro at a low frequency and produce subpatent infection in mice; parasites can be recovered by xenodiagnosis or hemoculture (55). Strain Y was isolated from a patient with an acute case of Chagas’ disease (42).

The parasites were maintained alternately in BALB/c mice and in liver infusion tryptose medium. Metacyclic forms, harvested from cultures at the stationary growth phase, were purified by passage through a DEAE-cellulose column, as previously described (46). Trypomastigotes derived from cell culture (TCTs) were collected in the extracellular medium from the seventh day of infection of CHO-K1 or Vero cells.

**Enucleation.** L929 cell monolayers on minislides were enucleated as described previously, with minor modifications (54). The concentration of cytocidalalin B used was 2.5 µg/mL, and cell monolayers were centrifuged for 30 min at 10,000 × g at 34°C. Under these conditions, about 50% of the cells were generally enucleated. Following centrifugation, minislides were washed once in Hanks’ saline, chased for 2 h in cDMEM at 34°C in a 5% CO₂–95% air atmosphere, washed, and infected with the parasite suspensions.

Survival of cytoplasts was reported to be extended markedly by culture at 31°C, washed three times with PBS, and permeabilized with 0.1% saponin (BDH, Amersham, United Kingdom) in PGN (PBS containing 0.2% gelatin and 0.1% NaN₃). Cells were then incubated with different MAbs (ascitic fluid diluted 1:50 in PGN) for 1 h at room temperature, washed three times with PBS, and developed with fluorescein-labeled goat anti-mouse IgG (Sigma), diluted 1:100 in PGN for 1 h, in the presence of 50 µM DAPI (4′,6′-diamidino-2-phenylindole; Molecular Probes, Eugene, OR). After three washes with PBS, minislides were mounted in glycerol buffered with 0.1 M Tris, pH 8.6, and 0.1% paraphenylene-diamine to reduce bleaching. Images were acquired with a Nikon E600 microscope with a Nikon Dxm1200 digital camera using ACT-1 software. Adobe Photoshop was used to pseudocolor the images. Fluorescence images of MAbs were generated in green, and DNA labeling is shown in blue.

**RESULTS**

Infective trypomastigotes obtained from axenic cultures, from the blood of infected animals, or from infected cell cultures enter nonprofessional phagocytes within phagolysosome-like vacuoles, where they begin to transform into amastigote forms prior to their release into the cytosol, where they multiply. In four or more days, multiplication ceases; amastigotes briefly acquire epimastigote-like morphology and markers and transform into noninfective trypomastigote forms which are cytolically released from the host cells (1, 2, 6, 26, 48). Serial observations of infections of bovine embryo skin and muscle cells have shown that prereplicative lag periods, doubling times of amastigotes, and durations of the entire intracellular cycle varied markedly between the five clones examined (12).

Infection of cytoplasts and nucleated cells as a function of time (qualitative observations). Figure 1 displays fluorescence and differential contrast interference images of cytoplasts and nucleated cells fixed at different times after a 5-h pulse with strain CL TCTs. It can be seen that after internalization, parasites differentiated into amastigotes, which multiplied in the cytosol; by 72 h and 96 h, epimastigote-like forms and trypomastigote-like forms developed and were characterized morphologically and immunocytochemically (Fig. 1), and parasites attached to cell surfaces were found in increasing numbers.

It was of interest to determine if the exit of parasites from the phagosomes took place similarly in cytoplasts and in nucleated cells. In the present experiments, nucleated cells and cytoplasts, present in the same preparations, were fixed 60 min, 90 min, 4 h, or 24 h after infection and stained for LAMP-1, a marker of *T. cruzi* parasitophorous vacuole membranes (33). Counts of cells containing parasites associated with the

**Antibodies.** Monoclonal antibodies (MAbs) 1D9 (immunoglobulin G3 [IgG3]) and 3B2 (IgG2a) were obtained from mice immunized with mixtures of heat-inactivated intracellular and extracellular amastigotes of the G strain. MAb 3B2 displayed high specificity for flagellated forms of the parasite, while MAb 1D9 was specific for the amastigote stage (3). Anti-LAMP-1 antibodies were anti-mouse LAMP-1 hybridoma supernatants from the Development Studies Hybridoma Bank (I.A.).

**Immunofluorescence.** Minislides containing infected cells were washed with phosphate-buffered saline (PBS) and fixed with 3.5% formaldehyde in PBS for 1 h; cultures were then washed three times with calcium- and magnesium-free PBS and permeabilized with 0.1% saponin (BDH, Amersham, United Kingdom) in PGN (PBS containing 0.2% gelatin and 0.1% Na₃). Cells were then incubated with different MAbs (ascitic fluid diluted 1:50 in PGN) for 1 h at room temperature, washed three times with PBS, and developed with fluorescein-labeled goat anti-mouse IgG (Sigma), diluted 1:100 in PGN for 1 h, in the presence of 50 µM DAPI (4′,6′-diamidino-2-phenylindole; Molecular Probes, Eugene, OR). After three washes with PBS, minislides were mounted in glycerol buffered with 0.1 M Tris, pH 8.6, and 0.1% paraphenylene-diamine to reduce bleaching. Images were acquired with a Nikon E600 microscope with a Nikon Dxm1200 digital camera using ACT-1 software. Adobe Photoshop was used to pseudocolor the images. Fluorescence images of MAbs were generated in green, and DNA labeling is shown in blue.
LAMP-1 marker revealed that at the two early time periods, for both nucleated and enucleated populations, nearly two-thirds of the infected cells contained parasites that colocalized with the LAMP-1 antigen. In contrast, in cells at both 4 and 24 h, the frequency of cells containing LAMP-1-associated parasites dropped to 4%. Thus, in both nucleated and enucleated cells, whereas by 60 or 90 min most parasites had not left their vacuoles, by 4 h essentially all were not associated with the phagolysosome marker (Fig. 2).

Percentages of infected cells, average numbers of parasites per cell, and frequency distributions of the numbers of parasites. Figure 3A displays the results of one of four independent experiments. In this experiment, about 70% of the cytoplasts and nucleated cells were infected with TCTs of strain CL, and the percentage of infection did not change significantly with time after infection. Counts of cytoplasts per area showed, however, that the total numbers of cytoplasts dropped with time, with no statistically significant difference between the numbers of infected and noninfected cytoplasts. Thus, taking the 12-h counts of total cytoplasts as 100%, the counts at 24, 48, and 72 h dropped to about 80%, 50%, and 20%, respectively (full results not shown).

The percentages of infection with strains CL-14, G, and Y were similar to or higher than those with strain CL, and likewise, statistically significant differences between nucleated and enucleated cells and different times of infection were not found (results not shown). Experiments using mtcyclic forms of strain CL showed that the percentages of infection were similar for cytoplasts and nucleated cells (results not shown).

Figure 3B shows that the average numbers of CL strain TCTs per cell were similar for nucleated and enucleated cells at 12 and 24 h of infection and increased significantly at 48 and 72 h; however, at the 48- and 72-h time points, the average numbers of parasites per cell were 50% higher for nucleated cells. Accordingly, frequency distributions of the numbers of parasites per cell demonstrated a progressive shift with time, from classes containing few to those containing large numbers.

FIG. 1. Nucleated (n) and enucleated (e) L929 fibroblasts infected with T. cruzi strain CL TCTs. Cell monolayers, kept at 34°C throughout, were enucleated, chased in complete medium, infected, washed, and fixed. Parasites in cells fixed 24, 48, and 72 h after infection were labeled with 1D9, an amastigote MAb. Cultures fixed at 96 h were labeled with 3B2, a trypomastigote MAb. In both cases, the primary antibodies were followed by a fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG secondary antibody. Nuclei were stained with DAPI. Arrows indicate intra-cellular parasites at 24 h. Bar = 10 μm for all panels.
of parasites (Fig. 4). Similar results were obtained with strains G and Y and with the CL-14 clone (results not shown). The total number of parasites in cytoplasts was estimated for each of the minislides by the following calculation: number of cytoplasts in the total area $\times$ percentage of infected cytoplasts $\times$ number of parasites per infected cytoplast. In the experiment shown in Fig. 3, at 12 and 24 h, prior to parasite multiplication, the total numbers of parasites in cytoplasts were $2.7 \times 10^5$ and $2.1 \times 10^5$, respectively. In contrast, at 48 and 72 h, these numbers increased to $3.1 \times 10^5$ and $4.8 \times 10^5$, respectively, even in the face of the marked loss of cytoplasts with time after enucleation.

In two separate experiments, the infection of 2-h-old cytoplasts was compared to that of cytoplasts aged 24 or 48 h. Cultures were fixed 5 and 24 h after infection. Counts revealed that percent infection and parasite counts were similar for the three groups of cytoplasts. In addition, after 24 h of infection, parasites were morphologically recognized as amastigotes (data not shown).

Doubling times of CL parasites in cytoplasts and nucleated cells. Plots of log$_2$ parasites per cell against time yielded parallel lines for cytoplasts and nucleated cells. The calculated doubling times for TCTs of the CL strain were 25.5 and 25 h for cytoplasts and nucleated cells, respectively. The doubling times for the other strains were 46.0 and 43.2 h for the CL-14 clone, 17.2 and 15.6 h for strain G, and 21.0 and 20.8 h for strain Y, respectively (Fig. 5).

**DISCUSSION**

We have shown that L929 cytoplasts can be productively infected with *T. cruzi* strain CL, up to and including the development of trypomastigotes. In both cytoplasts and nucleated cells, trypomastigotes left their parasitophorous vacuoles for the cytosol and differentiated into amastigote forms, as shown by their morphological and immunochemical features (Fig. 1). Numbers of intracellular amastigotes in both nucleated cells and cytoplasts remained stable for 12 and 24 h and increased significantly at 48 and 72 h of infection. At the late time points, however, although the estimated overall doubling times were not significantly different (Fig. 5), parasite loads were significantly lower in cytoplasts than in nucleated cells (Fig. 3B and 4). It should be noted that although the numbers of cytoplasts fell markedly with time of enucleation, the percentage of infected cytoplasts remained approximately constant. Thus, survival of cytoplasts does not appear to be either increased or reduced by infection with *T. cruzi*. Morphological and immunochemical observations indicated that by 48 h, and then more so by 72 and 96 h, many amastigotes differentiated into epimastigote- and trypomastigote-like forms. Finally, although extensive cell lysis and liberation of infective parasites were not demonstrated, the results suggest that cytoplasts could host a nearly complete infection cycle with *T. cruzi* parasites. Similar results were obtained with the CL-14 clone and with parasite strains Y and G (results not shown).

Interpretation of these findings requires consideration of the
biology of cytoplasts obtained from continuous cell lines, an area which has been relatively inactive for more than a decade. After mass enucleation of cells in monolayers was developed in the 1970s (9, 32, 53), early studies stressed that the behavior and ultrastructure of 12-h cytoplasts were similar to those of nucleated controls (17, 18, 19, 39, 40, 52). It was also shown, however, that soon after enucleation, protein synthesis assayed by the incorporation of radioactive precursors fell in cytoplasts kept at 37°C, to less than half of that in nucleated controls, and thereafter continued to decrease, reaching low levels at 12 and

![Graphs showing frequency distributions of TCTs of strain CL in infected nucleated and enucleated L929 cells at different times after infection.](http://iai.asm.org/)

FIG. 4. Frequency distributions of numbers of TCTs of strain CL in infected nucleated and enucleated L929 cells at different times after infection. Data shown are means ± standard errors of the means for the same experiment as that shown in Fig. 3. Time was a statistically significant source of variation ($P < 0.001$).

![Graphs showing intracellular multiplication of TCTs in infected cytoplasts and nucleated cells.](http://iai.asm.org/)

FIG. 5. Intracellular multiplication of TCTs in infected cytoplasts and nucleated cells. Enucleation, infection, staining, and counts were performed as described in Materials and Methods. Estimated doubling times were 25, 46, 16, and 21 h for strains CL, CL-14, G, and Y, respectively. Differences between nucleated cells and cytoplasts were not statistically significant ($P > 0.05$).
24 h (5, 15, 31, 44). By 24 h, cytoplasts were extensively vacuolated and thereafter displayed surface blebs, underwent fragmentation, and detached from the substrate. Although we confirmed that the survival of cytoplasts was extended by maintenance at 34°C, it is likely that in the course of infection parasites faced a progressively inadequate environment until the ultimate demise of the cytoplasts. Thus, pending additional information on the model, the more limited parasite loads in cytoplasts at 72 and 96 h postnucleation may be explained by the smaller size of these cells, the progressive decay of their metabolic activity, and/or the depletion of essential nutrients, such as l-proline (47). The possibility that at the latter infection points cytoplasts with high parasite loads died more rapidly than uninfected cytoplasts appears to be excluded by cytoplast counts per microscopic field.

Our results are compatible with the hypothesis that modulation of the transcription of host cell chromosomal genes is not obligatorily required in the course of *T. cruzi* infection. Furthermore, the observation that *T. cruzi* entry and differentiation into amastigotes can take place in 48-h-old cytoplasts suggests that short-lived host cell mRNAs, such as those that express AU-rich element sequences (8, 34, 50), may not be required during the early stages of infection. It may be of interest that a study of the transcriptional responses of normal human fibroblasts to infection with *T. cruzi* strain Y trypomastigotes revealed that modulation of transcription was negligible between 2 and 6 h postinfection but that a significant number of host cell genes were modulated by 24 h (49).

In earlier studies, cytoplasts were shown to be infected with the obligate intracellular pathogens *Toxoplasma gondii*, *Chlamydia psittaci*, *Chlamydia trachomatis*, and *Rickettsia prowazekii* (10, 20, 23, 29, 38, 45). Infection with the facultative intracellular *Toxoplasma gondii* (31) supports the role of host cell genes were modulated by 24 h (49).

REFERENCES


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