ENUCLEATED L929 CELLS SUPPORT INVASION, DIFFERENTIATION AND MULTIPLICATION OF *TRYPANOSOMA CRUZI* PARASITES.

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Abstract

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 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 in cytoplasts and nucleated cells. However, although 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 be also infected with 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 RNAs modification and processing in the nucleus.

**Introduction**

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 (15, 31). 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 non-viral 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, intracellular targeting, or modulate host cell functions required for survival, multiplication, and dissemination in the host (8, 12, 15, 26, 42).

It was also shown that, depending on the cell type and functional condition, host cell transcription can be, directly and/or indirectly, broadly modulated by prokaryotic or eukaryotic pathogens, their molecular components, and/or secreted products (15, 22, 23, 36). Some of these responses, common to viral and non-viral pathogens, were linked to stress, and to conserved, host defense mechanisms, whereas others were pathogen-specific (17, 22, 23). It may not be always 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 is 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 *T. cruzi* shares with other kinetoplastids (45), and its elaborate and relatively protracted intracellular life cycle (2, 7, 27, 49). 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., 2, 37, 38).
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 RNAs processing, thus disabling important cell nucleus-dependent signaling cascades (19). In principle, the infection of cytoplasts by a given pathogen may be unaffected, decreased or increased in comparison with their nucleated controls. Furthermore, if nucleated cells express transcription dependent microbicidal or microbistatic mechanisms (17), infection may be higher in enucleated than in nucleated host cells. In addition, if non-renewable cytoplasmic factors were required for \textit{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 \textit{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, in 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.

\textbf{Methods}

\textbf{Cells, media and growth conditions.} NCTC clone L929 fibrosarcoma 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/L bicarbonate and 15 mM HEPES [3-(N-morpholino) propanesulfonic acid]. Confluent cultures were trypsinized and \(1 \times 10^5\) cells seeded for 24 h in 2-cm\(^2\) wells, each containing an 8 x 12 mm “mini”-slide cut from standard microscope slides (54). Vero cells (African green monkey kidney fibroblasts) and CHO-K1 (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 \textit{T. cruzi} cycle.

\textbf{Parasites, media and growth conditions.} Four \textit{T. cruzi} strains were used in this study. Strain CL was originally isolated from \textit{Triatoma infestans} found in dwellings where people were infected (5); metacyclic trypomastigotes of this strain efficiently infect mice and invade mammalian cells in vitro. CL-14, a temperature-sensitive clone derived from 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 (25). Strain G was isolated from an opossum in the Brazilian Amazon metacyclic forms of this strain invade mammalian cells in vitro at low frequency and produce subpatent infection in mice; parasites can be recovered by xenodiagnosis or hemoculture (55). Strain Y was isolated from an acute case of Chagas' disease (44).

The parasites were maintained alternately in BALB/c mice and in liver infusion tryptose medium. Metacyclic forms (MTs), harvested from cultures at the stationary growth phase, were purified by passage through a DEAE-cellulose column, as previously described (47). Trypomastigotes derived of cell culture (TCTs) were collected in the extracellular medium from seventh day of infection of CHO-K1 or Vero cells.

**Enucleation.** L929 cell monolayers on mini-slides were enucleated as described, with minor modifications (54). The concentration of cytochalasin B used was 2.5 µg/ml, and cell monolayers were centrifuged for 30 min at 10,000 x g, at 34°C. Under these conditions, about 50% of the cells were generally enucleated. Following centrifugation, mini-slides 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 markedly extended by culture at 31°C, allowing for higher yields of temperature-sensitive or slow growing viruses to be obtained (16, 32). We confirmed that the survival of L929 cytoplasts kept at 34°C nearly doubles in comparison with those kept at 37°C, and used this condition to examine their infection by *T. cruzi*; it had been previously shown that certain strains the parasite undergo full development in host cells kept at 34°C (25).

Viability of the cytoplasts was examined by the accumulation of neutral red in acidified vesicles and by the ingestion of *Leishmania (L.) amazonensis* amastigotes. Although the numbers of cytoplasts fell with time, nearly all took up neutral red and parasites (Coimbra and Khusal, unpublished).

**Infection.** Monolayers on mini-slides, containing both nucleated and enucleated L929 cells were infected for 5 h at a multiplicity of 4.5 10⁶ to 6.0 10⁶ with *T. cruzi* infective forms in cDMEM. Cultures were then thoroughly washed and incubated at 34°C, in a 5% CO2/95% air atmosphere, in fresh cDMEM. Unless otherwise stated, cultures were fixed in methanol at 12, 24, 48, and 72 h of infection and stained with Giemsa.
**Measurement of the infection.** Infection was assessed by light microscope counts in monolayers fixed at different times in methanol and stained with Giemsa. At least 300 nucleated and enucleated cells in the same monolayers were scored at 400x magnification. Intracellular parasites were counted in at least 70 nucleated and 70 enucleated cells in each of 2 or 3 replicate slides per point or treatment. Results are expressed in averages ± standard errors. Nucleated cells and cytoplasts counts per field were estimated microscopically in preparations fixed at different times.

Frequency distributions were built in classes of 10 parasites per cell, with the frequencies normalized as a percent of the total numbers of nucleated or enucleated cells scored (54). Significances of the differences between averages with time were examined with the help of a Prism 4.0 Package using one or two ways ANOVA and the Bonferroni post-test.

To assess the exit of parasites from their parasitophorous vacuoles, cultures the presence of LAMP-1 antigen associated with the parasites was evaluated by immunofluorescence in cultures fixed at different times after infection. Between 30 and 60 infected cells were scored in each of 2 slides, for LAMP-1 associated parasites at different times after infection.

**Antibodies.** Monoclonal antibodies (MAb) 1D9 (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 (4). Anti-LAMP-1 antibodies were anti-mouse LAMP-1, hybridoma supernatants from Development Studies Hybridoma Bank (Iowa, USA).

**Immunofluorescence.** Mini-slides containing infected cells were washed with PBS and fixed with 3.5% formaldehyde in PBS for 1 h; cultures were then washed three times with calcium and magnesium free phosphate buffered saline (PBS) and permeabilized with 0.1% saponin (BDH, Amersham, UK) in PGN (PBS containing 0.2% gelatin and 0.1% NaN₃). Cells were then incubated with different MAb's (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 dihydrochloride, Molecular Probes, Eugene, Oregon). After three washes with PBS, mini-slides were mounted in glycerol buffered with 0.1 M Tris pH 8.6 and 0.1% paraphenylenediamine to reduce bleaching. Images were acquired in a Nikon E600 microscope with a Nikon DXM1200 digital camera using ACT-1 software. Adobe Photoshop was used to
pseudocolor the image. Fluorescence images of MAb were generated in green, and the DNA labeling in blue.

RESULTS

Infective trypomastigotes obtained from axenic cultures, from the blood of infected animals, or from infected cell cultures, enter non-professional phagocytes within phagolysosome-like vacuoles, where they begin to transform into amastigote forms prior to their release into the cytosol, where they multiply. By 4 or more days, multiplication ceases; amastigotes briefly acquire epimastigote-like morphology and markers and transform into non-dividing infective trypomastigote forms which are cytolytically released from the host cells (1, 2, 7, 27, 49). Serial observations of the infection of bovine embryo skin and muscle cells have shown that pre-replicative lag periods, doubling times of amastigotes, and duration of the entire intracellular cycles varied, markedly between the five clones examined (13).

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 5h pulse with tissue culture-derived strain CL trypomastigote forms (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 as in nucleated cells. In the present experiments, nucleated cells and cytoplasts, present in the same preparations, were fixed at 60 min, 90 min, 4 h or 24 h after infection, and stained for LAMP-1, a marker for T. cruzi parasitophorous vacuole membranes (34). Counts of cells containing parasites associated with the LAMP-1 marker revealed that, at the two early time periods, in both nucleated and enucleated populations, nearly 2/3rds of the infected cells contained parasites that colocalized with the LAMP-1 antigen. In contrast, in both cells at 4 and 24 h, the frequency of cells containing LAMP-1-associated parasites, dropped to less than 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).

**Percent 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 percent infection did not significantly change 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 non-infected 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 percent infection with strains CL-14, G and Y, was similar or higher than 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 MTs of strain CL showed that the percent infection was similar in cytoplasts and nucleated cells (results not shown).

Figure 3B shows that the average numbers of CL strain TCTs per cell were similar in 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 in nucleated cells. Accordingly, frequency distributions of the numbers of parasites per cell demonstrated the progressive shift with time, from classes containing few to those containing higher numbers of parasites (Fig. 4). Similar results were obtained with strains G, Y and with the CL-14 clone (results not shown). The total number of parasites in cytoplasts was estimated, in each of the mini-slides, by (numbers of cytoplasts in the total area) x (percent of infected cytoplasts) x (number of parasites per infected cytoplast). In the experiment shown in Figure 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 for 24 or 48 h. Cultures were fixed 5 and 24 h after infection. Counts revealed
that percent infection and parasite counts were similar in the three groups of cytoplasts. In addition, after 24 h of infection, parasites were morphologically recognized as amastigotes (data now shown).

**Doubling times of CL parasites in cytoplasts and nucleated cells.** Plots of numbers of parasites per cell in log base 2 against time yielded parallel lines for cytoplasts and nucleated cells. The calculated doubling times for TCTs of the CL strain were, respectively, 25.5 and 25 h for cytoplasts and nucleated cells. 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* of strain CL, up to and including the development of the 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 is to be noted that, although the numbers of cytoplasts fell markedly with time of enucleation, the percent 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 immunocytochemical observations indicated that by 48 h, and the 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 was 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 (10, 33, 53), early studies stressed that the behavior and ultrastructure of 12 h cytoplasts were similar to those of
nucleated controls (18, 19, 20, 40, 41, 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; thereafter it continued to decrease, reaching low levels at 12 and 24 h (6, 16, 32, 43). By 24 h, cytoplasts were extensively vacuolated and, thereafter, displayed surface blebs, underwent fragmentation, and detached from the substrate. Although we confirmed that 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 load in cytoplasts 72 and 96 h post enucleation 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 L-proline (48). 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 ARE sequences (9, 35, 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 Y strain trypomastigotes, revealed that modulation of transcription was negligible between 2 and 6 h post infection, but a significant number of host cell genes was modulated by 24 h (3).

In earlier studies cytoplasts were shown to be infected with the obligate intracellular pathogens Toxoplasma gondii, Chlamydia psittacci, C. trachomatis, and Rickettsia prowazekii (11, 21, 24, 30, 39, 46). Infection with the facultative intracellular bacterium, Shigella flexneri, was however, significantly reduced in L929 cytoplasts, and, as in the present results, the maximal bacterial loads attained were diminished in comparison to those in nucleated cells (54). It was also reported, on the basis of few observations, that T. cruzi amastigotes did not differentiate and replicate in HeLa cell cytoplasts (29). Although the results here reported support previous observations with a few other pathogens, we believe that the participation of the host cell nucleus in the infection should be examined in additional eukaryotic and prokaryotic
pathogens (28). It is to be noted that, after a more extensive screen, classic studies identified viruses which, contrary to then prevailing expectations, productively infected enucleated host cells (32). Furthermore, the results of the experiments may depend on the species and nature of the enucleated host cells, as suggested by the report that, whereas Sindbis virus grew in enucleated BHK-21 hamster fibroblasts, it did not infect enucleated cells from a mosquito cell line (14).

The mechanisms by which, in the course of evolution of intracellular parasitism, microbial infection may have become proximately independent of the host cell nucleus, remain to be investigated (51).

Acknowledgements

This work was supported by UNIFESP and by grants and scholarships from FAPESP and CNPq. Help from Sergio Schenkman, Kendi Okuda, Mariane B. Melo-Braga, Edgar J. P. Gamero, and Fernando Real is gratefully acknowledged. The authors wish to dedicate this paper in the memory of their direct or indirect teachers Leonidas Deane (1914-1993) and Maria von Paumgartter Deane (1916-1995).

References


**Figures**

**FIG. 1**. Nucleated (n) and enucleated (e) L929 fibroblasts infected with *T. cruzi* of strain CL cell culture derived trypomastigotes. Cell monolayers, kept throughout at 34°C, were enucleated, chased in complete medium, infected, washed and fixed. Parasites in cells fixed at 24, 48 and 72 h, after infection, were labeled with 1D9, a monoclonal antibody (amastigotes MAb). Cultures fixed at 96 h were labeled with 3B2, a monoclonal antibody (trypomastigotes MAb) at 96 h. In both cases, the antibodies were followed by a rabbit anti-mouse IgG-FITC labeled secondary antibody. Nuclei were stained with DAPI. Arrows indicate intracellular parasites at 24 h. Magnification bar = 10 µm for all panels. Nuclei stained with DAPI.
FIG. 2. Infection of L929 cytoplasts by *T. cruzi* strain CL parasites. A-C: Two hours after enucleation, cells were infected with TCTs for 1h, washed, chased for 15 min in complete medium and fixed. D-F: Cells similarly infected were washed and chased for 24h. A and D, DAPI; B and E anti-Lamp-1 antibody followed by FITC second antibody; C and F phase images of the same cells. Bar = 10µm. Arrows point to a parasite with Lamp-1 antigen, presumably localized to the phagosome membrane.

FIG. 3. Infection of L929 cytoplasts by TCTs of strain CL. Cells were enucleated and infected as described in Materials and Methods, fixed with methanol at different times and stained with Giemsa. (A) Percent of infection; (B) Numbers of parasites per cell. Asterisks in B indicate statistically significant differences (P < 0.001) of numbers of parasites in nucleated and enucleated cells, at 48 and 72 h compared those in the 12 h group (One way ANOVA, Bonferroni post-test). As indicated by the symbol # the number of parasites in nucleated cells was significantly higher than in cytoplasts (2-way ANOVA, Bonferroni post-test, P<0.001). The figure presents the results of one of five separate concordant experiments (means ± SEM).

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

FIG. 5. Intracellular multiplication of TCTs in infected cytoplasts and nucleated cells. Enucleation, infection, staining and counts were performed as in Methods of Procedure. Estimated doubling times were, respectively, 25, 46, 16 and 21 h for CL, CL-14, G and Y. Differences between nucleated cells and cytoplasts were not statistically significant (P >0.05).
The graphs show the relationship between time (in hours) and the number of parasites per cell for different conditions labeled CL, CL 14, G, and Y. The data points are differentiated by Enu (squares) and Nuc (triangles). The graphs demonstrate a linear increase in parasite load over time for both conditions.