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Laboratory techniques to obtain different forms of *Trypanosoma cruzi*: applications to wild-type and genetically modified parasites

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Abstract: Nowadays, there are no simple techniques for mimicking *in vitro* the life cycle of the kinetoplasmtid *Trypanosoma cruzi* Chagas, 1909, causative agent of Chagas disease, especially for parasite strains maintained as epimastigotes for many years. In the present study, we propose a method for obtaining metacyclic trypomastigotes, which were capable of infecting mammalian cells by simply lowering pH media. The collected amastigotes and trypomastigotes were differentiated into epimastigotes closing *T. cruzi* life cycle *in vitro*. Metacyclogenesis rates and infectivity were enhanced in cycled parasites. Finally, using this method, we were able to infect cells with transgenic parasites obtaining trypomastigotes and amastigotes using a neomycin-resistant cell line.

Keywords: Chagas disease, metacyclogenesis, parasite differentiation, intracellular epimastigotes

During its life cycle, the kinetoplastid *Trypanosoma cruzi* Chagas, 1909 suffers a wide variety of morphological and gene expression changes (Goldenberg and Avila 2011). The factors that trigger the differentiation process in *T. cruzi* are still unknown (Kolev et al. 2012). In nature, they occur spontaneously and can be mimicked *in vitro* by different techniques. However, very few of them keep the whole life cycle, returning to the epimastigote stage in each cycle.

One of the first method to differentiate epimastigotes to metacyclic trypomastigotes derives from the observation of Camargo (1964), in which about 10–15% of metacyclic forms appeared in epimastigote cultures only at the end of the exponential growth phase. The differentiation process did not occur if parasites were maintained in permanent exponential growth (Camargo 1964). A similar approach was also described by Dusanic 1980.

A few years later, Castellani et al. (1967) proposed a method which relied on lowering pH medium. A reduction from 7.2 to 6.7 produced about 40% of metacyclic forms, but this process was completely abolished if pH was reduced to 6.3 (Castellani et al. 1967). In contrast, Ucros et al. (1983) used a defined culture medium at pH 9 to produce metacyclic forms. The most widely used method was originally published by Contreras et al. (1985); this method consists of the incubation of culture - derived epimastigotes in triatomine artificial urine (TAU), which could be supplemented with proline (TAU-P). Later, more variants were developed, such as TAU supplemented with proline, glutamate, aspartate and glucose (TAU-3AG), which are effective for the differentiation of highly infective strains, but are not useful for laboratory cultures maintained for a long time as epimastigotes. Finally, other methods based on nutritional stress were also described (De Lima et al. 2008).

Several techniques have been developed for obtaining amastigotes *in vitro*. The process of amastigogenesis occurs when metacyclic trypomastigotes from triatomine urine differentiate into amastigotes inside mammalian host cells and when tissue-derived trypomastigotes invade new cells and differentiate newly to amastigotes. *In vitro* protocols for amastigote formation consist basically of the purification of tissue-culture trypomastigotes and amastigotes after disruption of infected Vero cells. In addition, amastigotes can be generated *in vitro* from tissue-culture-derived trypomastigotes (Tomlinson et al. 1995, Contreras et al. 2002, Navarro et al. 2003) and also spheromastigotes can be obtained from metacyclic trypomastigotes (Rondinelli et al. 1988).

The techniques mentioned above, which are vastly used nowadays, have two main limitations: (1) there is no reliable way to complete the life cycle *in vitro* (Kimura et al. 1978), which implies the transformation of amastigotes in epimastigotes (De Lima et al. 2008), and (2) the majority of methods of metacyclogenesis do not work with some strains maintained for a long time in culture. Here, we present a simple and flexible protocol for obtaining all the *T. cruzi* stages emulating the natural life cycle *in vitro*.

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MATERIALS AND METHODS

Parasites

Stock cultures of *Trypanosoma cruzi* epimastigotes of the CL Brener and Y strains were maintained axenically at 28 °C in Liver Infusion Triptose (LIT) or Brain Heart Triptose (BHT) media (pH 7), respectively, supplemented with 10% fetal calf serum, 100 U/ml penicillin, and µg/ml streptomycin (Camargo 1964). Transfected parasites were maintained in the same media containing 100 µg/ml G418. Parasites were counted in a Neubauer hemocytometer chamber.

In vitro differentiation and infection

Trypanosoma cruzi epimastigotes of the CL Brener and Y strains at stationary growing phase (15 days) were collected and grown in LIT and BHT medium at pH 4, pH 5.8 and TAU-P (190 mM NaCl, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 50 mM L-proline in 8 mM phosphate buffered saline (PBS) pH 6.0) for 6–8 days at 28 °C. Then, 2 ml of culture, containing 10^8 parasites (epimastigotes and trypomastigotes), were tested for cell infection using a monolayer of Vero cells (60% confluent), grown in MEM supplemented with 10% fetal calf serum. Twenty-four hours later parasites were removed and cells were maintained at 37 °C in MEM medium supplemented with 3% fetal calf serum, subsequently cells' medium was replaced every day. Infections were monitored periodically under the light microscope and the number of infected cells, amastigotes/cell and released trypomastigotes/cell were recorded. Infected cells were considered those containing intracellular amastigotes. Results are presented as the average of 200 cells analysed.

Trypomastigote forms were collected after cells disruption and used for reinfection and for recycling to epimastigote stage. For epimastigotes production, trypomastigotes and amastigotes were collected and cultured in LIT or BHT medium at pH 7. When the number of epimastigotes increased, they were subjected to differentiation at low pH conditions for reinfection or transfection assays. This cycle was repeated three times for the CL Brener strain and two times for the Y strain. The same procedure was followed for cycled transfected parasites. In that case, infection procedures were done in a G418 resistant Vero cells strain and selection was maintained during infection conditions.

Statistics

Data were analysed as follows: first normal distribution was tested using a Kolmogorov-Smirnov test and all groups presented a normal distribution. Afterwards, groups were analysed using a one-way ANOVA followed by a post-hoc Dunnet's multiple comparison test using a significance cut-off value of $P < 0.05$.

Fluorescence microscopy

Freshly grown trypanosome samples were washed twice in PBS. After letting the cells settle for 30 min at room temperature onto poly-L-lysine coated coverslips, parasites were fixed at room temperature for 20 min with 4% formaldehyde in PBS, followed by a cold methanol treatment for 5 min. Afterwards, all the samples were treated with anti-GFP (green fluorescent protein) antibody (Invitrogen, Carlsbad, California, USA) for 1 h, followed by secondary antibody incubation anti-rabbit (Vector Laboratories, Burlingame, California, USA) for 1 h. Slides were mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, California, USA).

Infected cells with transfected parasites were grown in 24-well plates. Seven day post-infection, they were washed three-times with PBS, followed by fixation at room temperature 20 min with 4% formaldehyde in PBS. Cells permeabilization was achieved by 15 min incubation with 0.01% (v/v) Triton X-100 after fixation. Afterwards, samples were treated as explained above. Cells were observed in an Olympus BX51 fluorescence microscope. Images were recorded with an Olympus XM10 camera and analysed with MBF ImageJ for microscopy bundle (National Institutes of Health, Bethesda, Maryland, USA).

Plasmid constructions and parasites transfection

The sequence coding for the full-length *T. cruzi* histone H2B (TcH2B) was cloned from genomic DNA using the following primers H2BF: 5'-CCATGGCCACCCCCAAAAGCTC-3'; H2BR: 5'-GGATCCACTAGAGGCGCTGCGACACCG-3' and fused to the 3' end of the GFP gene present in the pTEX-eGFP expression vector by digesting EcoRI/BamHI. The pTEX-eGFP plasmid was constructed by cloning the eGFP into the pTEX-TAP vector, kindly provided by Dr. Esteban Serra (Instituto de Biología Molecular y Celular de Rosario, Argentina). A total of 10^8 parasites of the CL Brener strain cycled once were grown in LIT medium at 28 °C, harvested by centrifugation, washed with PBS and resuspended in 0.35 ml of electroporation buffer (PBS containing 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$). The cell suspension was mixed with 50 µg of plasmid DNA in 0.2 cm gap cuvettes (Bio-Rad Laboratories, Hercules, California, USA). The parasites were electroporated with a single pulse of 400 V, 500 µF with a time constant of about 5 ms. Stable cell lines were achieved after 90 days of treatment with 500 µg/ml G418 (Calbiochem, Darmstadt, Germany) and supplementation of LIT medium with 30% SFB (Pereira et al. 2003).

G418 resistant Vero cells

Stable Vero lines resistant to G418 were obtained using a pcDNA3 plasmid (Invitrogen, Carlsbad, California, USA), lacking the CMV promoter by digesting BglII/BamHI followed by re-ligation. For cell transfection Lipofectamine (Invitrogen, Carlsbad, California, USA) was used. For each transfection assay 0.6 µg of DNA were mixed with 25 µl of MEM without serum or antibiotics and 25 µl of Lipofectamine was added. The mixture was incubated at room temperature for 20 min and then placed over a Vero cell monolayer (70% confluent) in MEM without serum for 6 hours at cells at 37 °C in a CO_2 incubator. Cells were washed and media were replaced by MEM 10% fetal calf serum. Twenty four hours later, G418 150 µg/ml was added. Cells were monitored under the light inverted microscope. Stable lines were obtained and maintained with 500 µg/ml of G418.

RESULTS AND DISCUSSION

Trypanosoma cruzi epimastigotes (10^7 cells) of the CL Brener and Y strains, maintained by subculturing as epimastigotes for years, were placed in 5 ml of LIT or BHT medium, respectively, at pH 4. Four days after inoculation, intermediate forms (epimastigote/trypamastigote) with high mobility compared to trypomastigotes, and metacyclic trypomastigotes were observed. Proportion of these parasite forms increased up to 30% and 65% on day 8, for CL Brener and Y strains, respectively. Similar treatments were also performed increasing media pH to 5.8 and also

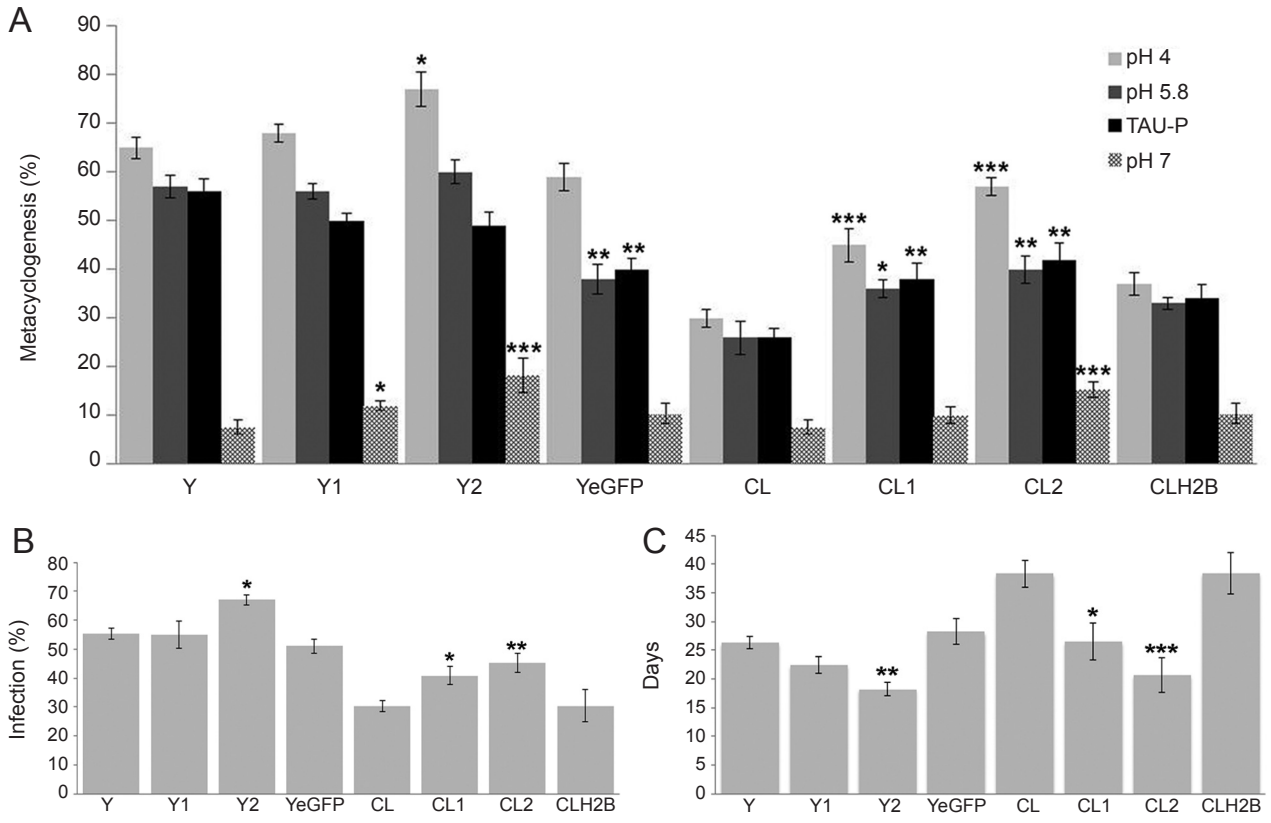

Fig. 1. Dynamics of *in vitro* cycling of staged of *Trypanosoma cruzi.* A – metacyclogenesis rates using neutral or low pH media or TAU-P. B – infection efficiency in Vero cells. C – time taken to complete a cycle. Names of individual strains – see the text. Significance levels were indicated with asterisks, and non-significant values were abbreviated NS.

in TAU-P. Interestingly, Y strain expressing the histone H2B showed a significant decrease in the metacyclogenesis rate at pH 5.8 and also in TAU-P. This phenomenon could be attributed to some effect associated to overexpression of the histone. In both media differentiation rates were slightly lower than those obtained at pH 4 (Fig. 1A).

Using LIT or BHT medium at neutral pH spontaneous metacyclogenesis continuously increases between cycles from 7.7% (SD ± 0.9) to 18.3% (SD ± 3.2, Y2) and from 7.5% (SD ± 1.1, CL0) to 15.3% (SD ± 1.5, CL2).

Infection assays were performed 8 days after culture at pH 4. The first trypomastigotes were observed about 20 days post-infection in both strains, without subculturing. The optimal period of time for infective forms collection was 4–12 days after infection. Amastigotes were obtained by leaving the infected culture for 3–4 days (until 12–16 day post-infection) at 37 °C without replacement of media.

When a large number of trypomastigotes (about 10^7 cells) were released, they were collected and placed in 5 ml of LIT or BHT medium at pH 7. Parasites were grown without subculturing and were observed periodically under the light microscope. Initially, they adopted a round shape and agglutinated forming of groups parasites groups; afterwards, they started to develop flagella and change to epimastigotes. The first epimastigotes were observed around 30 days post-inoculation. When parasites reached a density of approximately 10^7 cells/ml, subcultures were performed. After several passages in LIT or BHT pH 7, they started growing similarly as cultured epimastigotes. The whole procedure described up to this point is what was called the 'first cycle' (Y1 and CL1).

Cycled epimastigotes (10^7 cells) of both strains (Y1 and CL1) were transferred to low pH media (pH 4), for posterior infection repeating the above protocol, starting the 'second cycle' (Y2 and CL2). Comparing with previous cycles, the number of metacyclic trypomastigotes showed a significant increase and, additionally, infection times presented a significant decrease (Fig. 1C). Re-infection and cycling times diminished in each additional cycle. This might be because of selective pressures; the most virulent parasites are probably selected in each round of infection. The high differentiation percentages obtained for the second cycle of the CL Brener strain (about 60% after ten days of culture in LIT medium at pH 7) reinforce this hypothesis. There was no need for low pH treatment for metacyclogenesis and these parasites were capable of infecting cells in 14 days. Both strains improved the metacyclogenesis rate (Fig. 1A), infectivity (Fig. 1B) and cycling time (Fig. 1C) after each cycle. Additional cy-

Table 1. Rate of trypomastigotes and amastigotes of *Trypanosoma cruzi* per cell.

Trypomastigotes/cell

	Mean ± SD	Y1	Y2	YH2B	CL0	CL1	CL2	CLeGFP
Y0	0.21 ± 0.07	NS	***	NS	NS	-	-	-
Y1	0.27 ± 0.08		-	-	-	NS	-	-
Y2	0.45 ± 0.11			-	-		***	-
YH2B	0.17 ± 0.04				-	-	-	NS
CL0	0.17 ± 0.05					***	***	NS
CL1	0.29 ± 0.08						-	-
CL2	0.32 ± 0.08							-
CLeGFP	0.18 ± 0.06							

Amastigotes/cell

	Mean ± SD	Y1	Y2	YH2B	CL0	CL1	CL2	CLeGFP
Y0	16.3 ± 2.2	***	***	NS	**	-	-	-
Y1	20.7 ± 2.8		-	-	-	NS	-	-
Y2	26.0 ± 1.8			-	-		***	-
YH2B	13.7 ± 2.5				-	-	-	NS
CL0	19.7 ± 3.0					***	***	NS
CL1	21.8 ± 2.1						-	-
CL2	15.3 ± 2.2							-
CLeGFP	15.2 ± 1.9							

NS not significant; SD standard deviation; ** $p < 0.05$; *** $p < 0.005$.

cles further increase the mentioned parameters (data not shown). Subcultured epimastigotes that were previously submitted to this cycling protocol retained their enhanced infectivity for at least six months.

To obtain different *T. cruzi* stages from transgenic parasites, cycled epimastigotes of the CL Brener and Y strains were transfected with standard *T. cruzi* expression plasmids. To evaluate their capacity of expressing and targeting proteins to subcellular organelles in infective stages, we used the pTEX-H2B::eGFP and pTEX-eGFP vectors expressing the histone H2B fused to GFP (nuclear marker) and GFP alone (cytosolic marker). After selection parasites were challenged to low pH conditions. The number of metacyclic trypomastigotes was similar to the ones observed for non-transfected parasites. In infection assays, the first trypomastigotes were observed 30 days post-infection. Both trypomastigotes and amastigotes from the CL Brener and Y strains expressed fluorescent proteins (Fig. 2A,B).

Posterior re-infections, in which similar transgene expression levels were maintained, were achieved. When the amount of trypomastigotes was considerably high, they were shifted to LIT medium pH 7 and, 30 days post-inoculation, the first epimastigotes, which still expressed the transgenes, were observed. Furthermore, Fig. 2C shows CL Brener parasites transfected with pTEX-H2B::eGFP, presenting an unusual morphology obtained 4–6 days post-infection between the trypomastigote/amastigote transition. This intermediate stage is similar to the previously described 'intracellular epimastigote-like form' (Almeida-de-Faria et al. 1999).

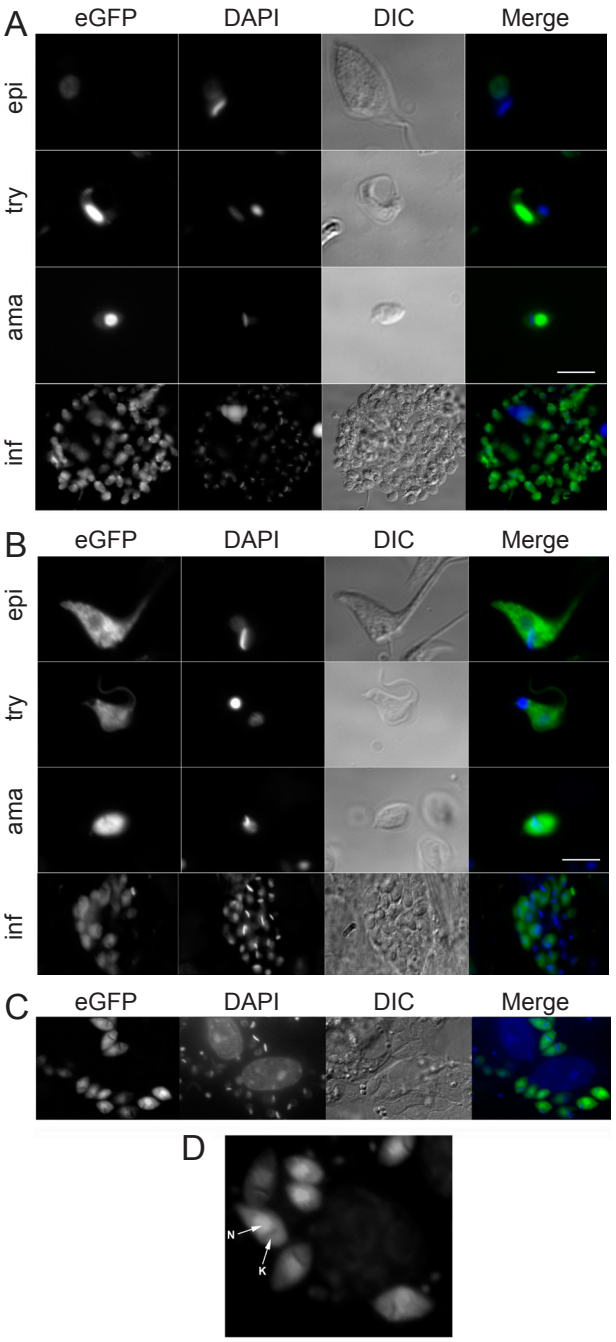

Fig. 2. Differentiation and infection using eGFP::H2B and eGFP transgenic parasites. CL Brener parasites transfected with the pTEX-eGFP::H2B (**A**) or Y strain parasites expressing the pTEX-eGFP (**B**) were analysed by fluorescence microscopy. Green fluorescence protein (eGFP), 4',6-diamidino-2-phenylindole (DAPI), differential interference contrast (DIC), and the merged image (Merge) shown in epimastigotes (epi), trypomastigotes (try), amastigotes (ama) and infected Vero cells (inf). **C** – Intracellular epimastigotes-like forms obtained at 4–6 days post-infection of Vero cells with CL Brener parasites carrying the pTEX-eGFP::H2B construction. **D** – magnification of intracellular epimastigotes; the typical nuclear (N) and kinetoplast (K) organization of epimastigote forms is highlighted (arrows). Scale bars in the corner of the merged images = 5 μm.

Finally, the number of trypomastigotes and amastigotes per cell were calculated. As Table 1 shows, a significant increase was observed in the amount of trypomastigotes and amastigotes per cell, when compared to controls (cycle 0), in both parasite strains during the cycle 2, and only in the Y strain during the cycle 1. On the other hand, the Y strain has shown a significant increase in the amount of trypomastigotes and amastigotes per cell after the second cycle when compared with the CL Brener strain.

In conclusion, we were able to mimic *T. cruzi* life cycle *in vitro* using a simple method. Parasite recycling enhanced infectivity due to possible natural selection pressures. Furthermore, this method allows infection with transgenic parasites, which is a very powerful tool for studying protein localization and function in the infective parasite stages. In addition, fluorescent parasites can be used to monitor and record infection *in vivo* under a fluorescence microscope, allowing further advances in the understanding of the invasion process. Finally, in transgenic parasites where gene over-expression is partially toxic, antibiotic selection pressure must be maintained. For this reason, our design allows the use of antibiotics at every stage of the life cycle as a consequence of achieving infections in a G418 resistant Vero cell line. Nowadays, most of the functional genomics research on *T. cruzi* is made in epimastigote cells, mainly for technical simplicity of their culture and manipulation. We expect that the presented methodology allows and encourages experimentation with trypomastigotes and amastigotes, the most relevant life cycle stages from a therapeutic point of view.

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