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Cell culture and animal infection with distinct *Trypanosoma cruzi* strains expressing red and green fluorescent proteins

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Abstract

Different strains of *Trypanosoma cruzi* were transfected with an expression vector that allows the integration of green fluorescent protein (GFP) and red fluorescent protein (RFP) genes into the β-tubulin locus by homologous recombination. The sites of integration of the GFP and RFP markers were determined by pulse-field gel electrophoresis and Southern blot analyses. Cloned cell lines selected from transfected epimastigote populations maintained high levels of fluorescent protein expression even after 6 months of in vitro culture of epimastigotes in the absence of drug selection. Fluorescent trypomastigotes and amastigotes were observed within Vero cells in culture as well as in hearts and diaphragms of infected mice. The infectivity of the GFP- and RFP-expressing parasites in tissue culture cells was comparable to wild type populations. Furthermore, GFP- and RFP-expressing parasites were able to produce similar levels of parasitemia in mice compared with wild type parasites. Cell cultures infected simultaneously with two cloned cell lines from the same parasite strain, each one expressing a distinct fluorescent marker, showed that at least two different parasites are able to infect the same cell. Double-infected cells were also detected when GFP- and RFP-expressing parasites were derived from strains belonging to two distinct *T. cruzi* lineages. These results show the usefulness of parasites expressing GFP and RFP for the study of various aspects of *T. cruzi* infection including the mechanisms of cell invasion, genetic exchange among parasites and the differential tissue distribution in animal models of Chagas disease.

Keywords: *Trypanosoma cruzi*; Green fluorescent protein; Red fluorescent protein; Chagas disease

1. Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is one of the major public health problems in Latin America where 16–18 million people are affected (World Health Organization, http://www.who.int/ctd/chagas/disease.htm). The disease usually presents an acute phase characterized by high parasitemia and a variable clinical course, ranging from asymptomatic to severe chronic cardiac and/or gastrointestinal disease. The reasons for such variation in both the severity and prevalence of the diverse clinical forms of the disease are not understood, but have been attributed to differences in the human genetic background and to the highly diverse genetic profile of the parasite population. In spite of its extreme genetic variability, several molecular markers have defined two highly divergent sub-groups of strains, named *T. cruzi*I and II, in the *T. cruzi* population (for a review, see Macedo et al., 2004). Recently, the existence of a third ancestral lineage named *T. cruzi* III has been proposed (Freitas et al., 2006).

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During development in the vertebrate host, the number of detectable parasites rapidly decay and in most cases their detection in infected tissues during the chronic phase can only be monitored through sensitive immunohistochemistry techniques (Andrade and Andrews, 2004; Souto-Padron et al., 2004; Correa-de-Santana et al., 2006) or by PCR amplification of parasite DNA sequences (Andrade et al., 1999; Vago et al., 2000; Freitas et al., 2005). PCR amplification also allows the characterization of the genetic profiles of different strains isolated directly from cardiac and megaesophagus tissues of chagasic patients (Vago et al., 2000; Freitas et al., 2005). In animal models of infection, the use of Low-Stringency Single Specific Primer (LSSP)-PCR permitted the identification of a differential tissue distribution when distinct cloned parasite strains were simultaneously inoculated, thus reinforcing the importance of *T. cruzi* genetic polymorphisms in determining the pathology of Chagas disease (Andrade et al., 1999).

As an approach to detect intact *T. cruzi* in infected animals, various groups have developed genetically modified strains capable of expressing different markers. Using the *Escherichia coli* β-galactosidase gene, Buckner et al. (1999) generated transfected cell lines that allowed the detection of *T. cruzi* in mouse tissues by histochemical staining with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal). However, besides requiring laborious tissue fixation and X-Gal staining, this method does not allow study of the infection dynamics of living, β-galactosidase-expressing parasites. Transfection of epimastigotes with various vectors containing the jellyfish green fluorescent protein (GFP) gene has resulted in GFP expression in all three stages of the parasite. In the first report, the vector used contains the GFP gene linked to the amastin 3′ untranslated region (UTR), which confers increased stability of amastin mRNA in amastigotes, and the construct was targeted to the *tcr* 27 locus (Teixeira et al., 1999). The GFP-containing vector described by Dos Santos and Buck (2000), pPAGFPAN, is present in stably transfected parasites in at least three forms: single copy, head-to-tail concatemers and integrated in multiple loci in the parasite genome. Using the pTEX vector, which is also episomally maintained, Ramirez et al. (2000) described the use of GFP to improve transfection protocols in *T. cruzi*. More recently, we developed the pROCKNeo vector, designed to allow integration of a foreign gene by homologous recombination into the tubulin locus, which contains a large array of alternating copies of α- and β-tubulin genes. This vector also contains sequences derived from the flanking regions of the *T. cruzi* glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) genes, allowing constitutive expression of the foreign gene in all parasite forms (DaRocha et al., 2004).

Using the pROCKNeo vector, we describe here the generation of different *T. cruzi* strains stably expressing GFP or red fluorescent protein (RFP). When analyzed using fluorescence or confocal microscopy, green and red fluorescent parasites were easily observed in single or double-infected tissue culture cells, as well as in blood and various tissues of infected animals. These fluorescent parasites constitute a powerful new tool for in vivo studies addressing key issues for the understanding of Chagas disease pathology such as parasite invasion mechanisms, differential tissue tropism and mechanisms of genetic exchange that may contribute to create the large genetic variability in the *T. cruzi* population.

2. Materials and methods

2.1. Parasite cultures

Epimastigote forms of different *T. cruzi* strains, Tulahuén and JG (both from the *T. cruzi* II lineage), the Col1.7G2 clone derived from the Colombiana strain (belonging to *T. cruzi* I lineage) and CL Brener (a hybrid cloned strain) were grown in liver infusion tryptose (LIT) medium supplemented with 10% FCS at 28 °C as described by Camargo (1964). Tissue culture trypomastigotes were obtained after infecting Vero cell monolayer cultures with stationary phase epimastigotes grown in LIT medium.

2.2. Parasite transfections

Two vectors were used to generate stable transfectants. The first, pROCKGFPNeo, contains the GFP coding region and sequences that allow DNA integration at the β-tubulin loci (DaRocha et al., 2004). The second vector, pROCKRFPNeo, was engineered to replace the GFP coding region by RFP, derived from the pDsRed vector (Clontech), into the *Xba*I/*Xho*I sites of pROCKGFPNeo. Epimastigotes growing at a density of $1–2 \times 10^7$ parasites/mL in LIT plus 10% FCS were harvested, washed once with PBS and resuspended to a final density of 10^8 parasites/mL in electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM 4-(2-hydroxyethyl)-1-piperazi-neethanesulphonic acid (HEPES), 2 mM EDTA, 5 mM $MgCl_2$, pH 7.6). Aliquots of 0.4 mL of cell suspension were mixed with 50 μL of DNA (50 μg) in ice-cold 0.2 cm cuvettes and electroporated using a Bio-Rad pulser set at 0.3 kV and 500 μF with three pulses (10 s intervals between pulses). In order to generate stable transfectants, parasites were electroporated with *Not*I linearized DNA plasmids and 24 h after transfection, 200 μg/mL of the aminoglycoside antibiotic G418 (Invitrogen) was added to the medium. Drug selection was completed 30 days after electroporation as monitored by the number of surviving parasites in mock-transfected cultures (DaRocha et al., 2004).

2.3. Selection of GFP and RFP positive T. cruzi clones

After selection of recombinant *T. cruzi,* cloned sub-populations were obtained by serial dilution in 96-well plates containing LIT medium supplemented with 10% fresh human blood and 200 μg/mL of G418. Parasite numbers

were determined by counting cells with a Neubauer chamber and then serially diluted to a final density of 0.1 and 0.2 cells/well in a total volume of 0.2 mL. Parasites in 96-well plates were incubated at 28 °C in humid chambers for 3–4 weeks and analyzed with a fluorescence microscope. GFP- and RFP-positive parasites were transferred into 1 mL LIT medium supplemented with 200 μg/mL of G418 in 24-well tissue culture plates and incubated at 28 °C. Alternatively, transfected parasite populations were sub-cloned in blood-agar plates prepared using 0.75% (w/v) low melting point (LMP) agarose and 2% of defibrinated human blood mixed with LIT-medium containing 200 μg/mL of G418. Defibrinated blood was obtained by collecting blood in a sterile glass bottle containing glass beads that were manually rotated. After pouring into Petri dishes, the medium was allowed to solidify and the plates were inverted and incubated at 37 °C for 48 h to dry (Gomes et al., 1991). Different numbers of parasites (10^2, 10^3 and 10^4) diluted in 0.1 mL of LIT medium were spread over the agar surface with a sterile glass loop and incubated at 28 °C. After 25–30 days, isolated colonies could be observed on the agarose surface and individual colonies were picked and transferred into 1 mL of LIT medium supplemented with G418 in a 24-well tissue culture plate.

2.4. Pulse-field gel electrophoresis (PFGE), Southern and Northern blot analyses

Epimastigotes were embedded in agarose blocks as described by Engman et al., 1987 and pulse-field gel electrophoresis (PFGE) was carried out as reported by Cano et al., 1995 with the following modifications: the chromosomes were separated in 0.8% agarose gels using a program with five phases of homogeneous pulses (N/S, E/W) for 135 h at 83 V interpolation. Phase 1 has a pulse time of 90 s (run time 30 h); phase 2, 200 s (30 h); phase 3, 350 s (25 h); phase 4, 500 s (25 h) and phase 5, 800 s (25 h). Chromosomes from *Hansenula wingei* (Bio-Rad) were used as molecular mass standards. Separated chromosomes were transferred to nylon filters and hybridized with [^{32}P]-labeled tubulin and RFP probes as described (DaRocha et al., 2004). For northern blot analyses, total RNA was extracted from 5×10^8 epimastigotes using the RNeasy Mini Kit (QIAGEN), following the procedure recommended by the manufacturer. Ten micrograms of RNA were fractionated on 1.5% denaturing agarose–formaldehyde gels and transferred to nitrocellulose membranes following standard protocols (Teixeira et al., 1994). Hybridization was performed as described above for Southern hybridization.

2.5. Cell culture infections

Approximately 10^5 Vero cells were plated in 24-well plates containing glass coverslips. Vero cell infection was performed for approximately 18 h at 37 °C at a multiplicity of infection (MOI) of 10, using trypomastigotes from each *T. cruzi* population (transfected or non-transfected controls) that were collected from the supernatant of previously infected Vero cell cultures. After infection, cell cultures were washed with PBS to eliminate non-internalized parasites and re-incubated with fresh media without parasites for 6 days before fixation with methanol at −20 ° C. Fixed cultures were stained with Giemsa and May Grunwald according to Giaimis et al. (1992), and the coverslips mounted onto glass slides for microscopic analysis. Cell infection and the number of amastigotes per cell were evaluated by counting 300 Vero cells per coverslip for each parasite population. Three independent experiments were performed for each infection.

2.6. Infection in mice, preparation and staining of mouse tissues

Four to 6 week old, male, IFN-γ knockout (GKO) mice were inoculated i.p. with 100,000 culture-derived trypomastigotes from each transfected *T. cruzi* population in a volume of 200 μL. Parasitism was quantified beginning on day 7 p.i. by examining 5 μL of tail blood at a magnification of 40×. Mouse infections were monitored daily. At the peak of parasitemia, hearts, diaphragms and rectums of infected animals were collected, washed in PBS (pH 7. 2), embedded in Tissue-Tek O.C.T compound (Miles, Inc., Elkhart, Ind.) and frozen at −70 °C until used for sectioning. Ten micrometer thick frozen sections were prepared with a cryostat microtome (Bright Instrument company LTDA), mounted on glass slides and fixed with 4% paraformaldehyde in PBS for 10 min at 25 °C. Slides containing tissue slices were stained with 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 1 min at room temperature, washed three times with PBS and mounted onto glass slides in a mixture of 90% glycerol and 10% of 0.5 M Tris–HCl pH 9.0. Animal infection protocols were approved by the local animal ethics committee.

2.7. Confocal microscopy analyses

For confocal microscopic analyses, coverslips containing infected Vero cells were washed twice in cold PBS supplemented with 3% FCS and fixed for 10 min at 25 °C with 4% paraformaldehyde in PBS. After fixation, cells were washed twice in PBS supplemented with 3% FCS, stained with DAPI for 1 min at room temperature, washed three times with PBS and mounted onto glass slides in a mixture of 90% glycerol and 10% of 0.5 M Tris–HCl pH 9.0. Parasite fluorescence was detected in a Zeiss LSM 510 META confocal microscope provided by the Electron Microscopy Center (CEMEL-ICB/UFMG).

2.8. Flow cytometry analyses

Approximately 5×10^6 parasites were washed with PBS supplemented with 3% FCS and MFF (0.05% paraformaldehyde, 0.05% sodium cacodylate and 0.033% NaCl) for

30 min at 4 °C. Fixed parasites were analyzed using a FACScan (Becton Dickinson) with 10^5 gated events acquired for analysis. Untransfected control parasites, which showed a significant amount of intrinsic fluorescence, were used to establish cut-off values for the analyzed parameters.

3. Results

3.1. Characterization of transfected T. cruzi cell lines expressing GFP/RFP genes

Epimastigote cultures of the Tulahuén, JG, Colombiana and CL Brener strains were transfected with linearized pROCKGFPNeo or pROCKRFPNeo vectors. These vectors contain GFP or RFP expression cassettes flanked by β-tubulin sequences, which allow integration by homologous recombination into the tubulin locus of the parasite genome (Fig. 1a). After electroporation, epimastigotes were cultivated in media containing 200 μg/mL G418 for 4 weeks, i.e., until no parasites could be detected in mock-transfected cultures. G418-resistant parasites were cultivated through weekly passages in the presence of the drug and the percentages of fluorescent parasites were determined by fluorescent microscopy. One month after transfection, we observed 22% and 12% of transfected parasites of the JG strain expressing GFP and RFP, respectively. For the Tulahuén strain, 28% and 25% of parasites were expressing GFP and RFP, respectively. The CL Brener clone presented 30% and 22% GFP- and RFP-expressing parasites, respectively, whereas the Col1.7G2 clone presented 23% and 9% GFP- and RFP-expressing parasites, respectively. For all transfected strains, biological parameters including parasite mobility, morphology (form, size and granularity) and growth rate were comparable to untransfected, wild type (WT) *T. cruzi* populations, indicating that the transfection process and genetic recombination within β-tubulin loci had not altered the basic features of these parasites (data not shown).

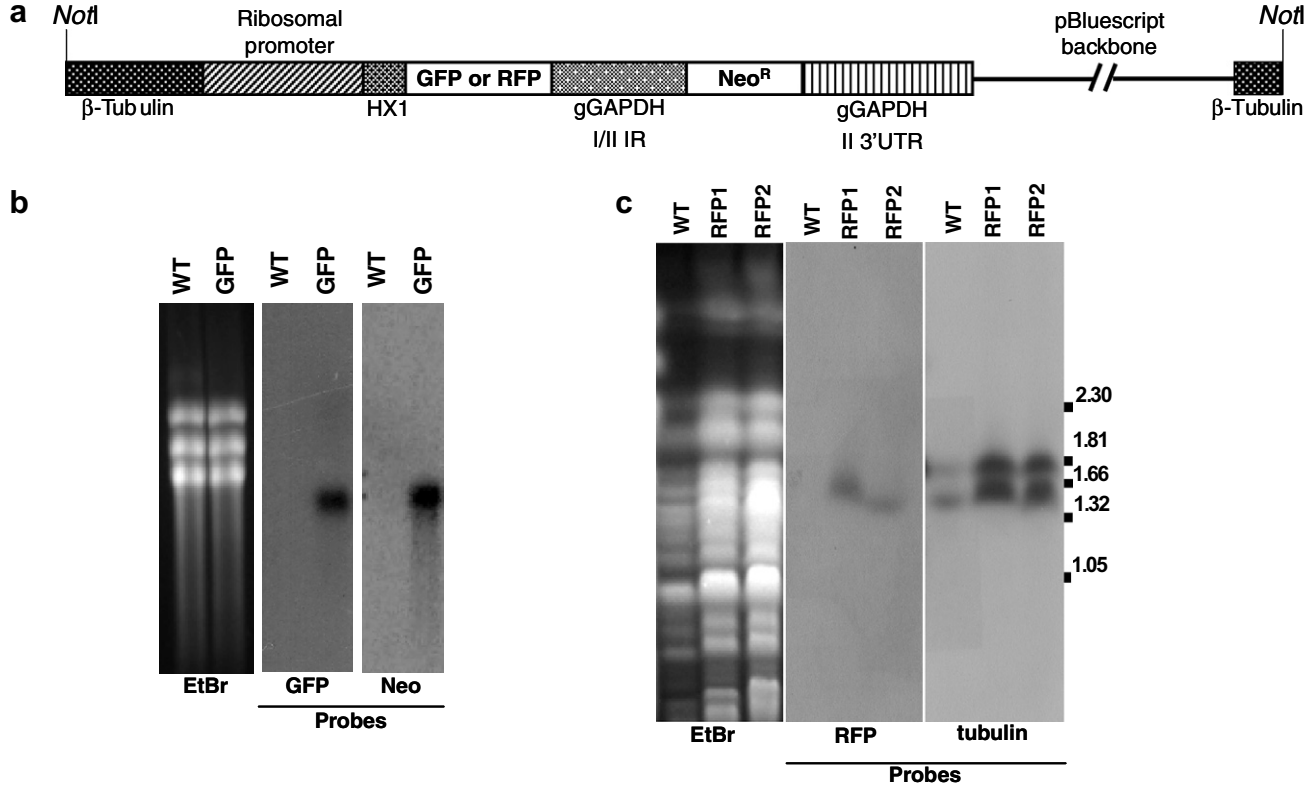

Fig. 1. Integration and mRNA expression of green/red fluorescent protein (GFP/RFP) genes following parasite transfection. (a) Schematic representation of the pROCKGFP/RFPNeo vector construct. The expression vector, which allows integration of a foreign gene in the β-tulubin locus, has the *Trypanosoma cruzi* ribosomal promoter followed by the ribosomal protein TcP2β (HX1) 5′ intergenic region that provides the spliced leader addition site for the GFP/RFP mRNA. It also contains the 3′UTR plus intergenic sequences derived from the gGAPDH I/II genes, which provide signals for polyadenylation of the GFP/RFP genes as well as for trans-splicing signals for the neomycin resistance gene (Neo^R), used as a drug selectable marker. (b) Expression of GFP and Neo^R transcripts. Northern blot analyses were performed with total RNA extracted from wild type parasites (WT) and epimastigotes of the Tulahuén strain that had been transfected with pROCKGFPNeo (GFP). After separation in agarose gels, RNA samples were stained with ethidium bromide (left panel), transferred to a nitrocellulose membrane and hybridized with [^{32}P] labeled probes corresponding to the GFP and the Neo^R coding region. (c) Southern blot analyses of transfected parasites. High molecular weight DNA, isolated from WT cells and two cloned cell lines derived from epimastigotes of the JG strain that were transfected with pROCKRFPNeo (RFP1 and RFP2) was separated by PFGE and stained with ethidium bromide (EtBr). After transfer to nylon membranes, DNA samples were hybridized with [^{32}P] labeled probes corresponding to RFP and β-tubulin coding regions. Numbers on the right correspond to the sizes of chromosomal molecular weight markers.

Transfected parasite populations were cloned by serial dilution followed by plating in blood-agar medium. Two clones expressing GFP, TulaGFP1 and TulaGFP2, both with 40% of the parasites presenting green fluorescence and two clones of the Tulahuén strain expressing RFP, TulaRFP1 and TulaRFP2, in which 40% and 70%, respectively, of the parasites displayed red fluorescence were selected. After another round of cloning in blood-agar plates, we isolated two new sub-clones derived from TulaGFP2 and TulaRFP1 in which 95% and 84% of the parasites displayed green and red fluorescence, respectively. We also obtained, after one step of serial dilution cloning, two clones of the JG strain, named JGRFP1 and JGRFP2, in which 88% and 70%, respectively, of the parasites expressed RFP. As shown in Fig. 1b, expression of GFP and the Neo resistance mRNAs in the transfected cell lines was confirmed by Northern blot analyses. The sites of integration of the transfected DNA into the parasite genome were also determined by PFGE analyses (Fig. 1c). DNA from two clones derived from the JG strain transfected with pROCKRFPNeo co-hybridizes with chromosomal bands that are recognized by the RFP and β-tubulin probes, thus indicating that the RFP gene was integrated at the tubulin locus in homologous chromosomes presenting different sizes. Using a GFP probe, we have previously shown that GFP-expressing Tulahuén and Col1.7G2 clones also presented hybridization signals compatible with vector integration in the tubulin locus (DaRocha et al., 2004). The stability of the fluorescent markers in the transfected populations and clones, was evaluated after cultivation for 1 or 2 months in the absence or in the presence of 200 or 400 μg/mL of G418. Flow cytometry analyses showed that, in contrast with the transfected populations, in which a decrease of the percentages of fluorescent parasites occur, the percentages of fluorescent cells within the clones were constant even after 2 months of culturing without drugs. Also, increasing the G418 concentration from 200 μg/mL to 400 μg/mL did not cause significant changes in the fluorescent protein expression ratios (see Supplementary Fig. S1).

3.2. Cell culture and animal infection with transfected parasites

Next, fluorescent protein expression was evaluated in the mammalian forms of the parasite life cycle by infecting Vero cell cultures. Similar to epimastigotes, transfected parasites exhibited intense green or red fluorescence in the trypomastigote and amastigote stages (Fig. 2). Transfected parasites were not only able to infect Vero cells, but trypomastigotes and amastigotes maintained high levels of fluorescence even after 6 months of passage through tissue cell culturing without G418. None of the transfected clones presents a population of cells which were 100% fluorescent. However, similar to epimastigotes, non-fluorescent trypomastigotes and amastigotes were observed in Vero cells. To determine whether genetic modification has altered the infection capacity of the transgenic parasites, we added equal numbers of WT and transfected trypomastigotes obtained from previously infected Vero cells, to freshly plated host cells. Six days after infection, the numbers of infected cells, as well as the average number of intracellular amastigotes in each cell, in a total of 300 Vero cells were determined (Fig. 3). We found no significant dif-

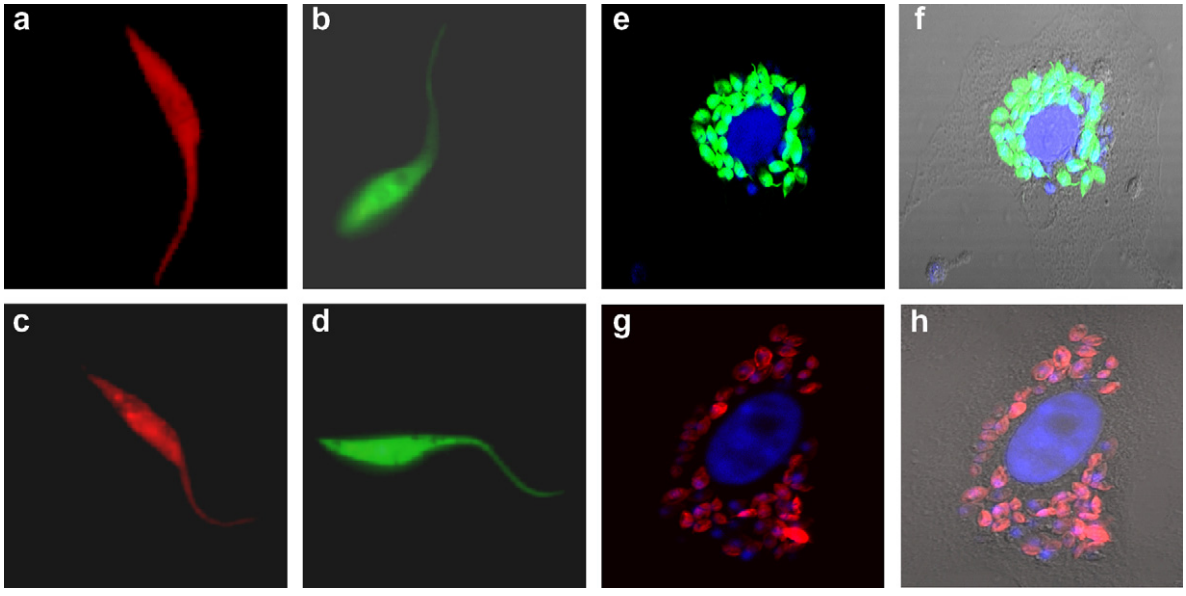

Fig. 2. Expression of green/red fluorescent proteins (GFP/RFP) in *Trypanosoma cruzi*. Cloned, transfected epimastigotes of the Tulahuén (a), JG (b), CL Brener (c) and Colombiana (d) strains expressing RFP (a and c) or GFP (b and d) were visualized under a fluorescent microscope at 60× magnification. Vero cells were incubated with parasites from stationary-phase cultures of two clones of transfected Tulahuén strain, TulaGFP2 (e and f) and TulaRFP1 (g and h) and stained with DAPI 6 days p.i. (e and g). Infected cells containing green and red intracellular amastigotes, respectively, visualized at 40× original magnification with a Zeiss LSM 510 META confocal microscope. (f and h) Merged fluorescence and differential interference contrast (DIC) images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

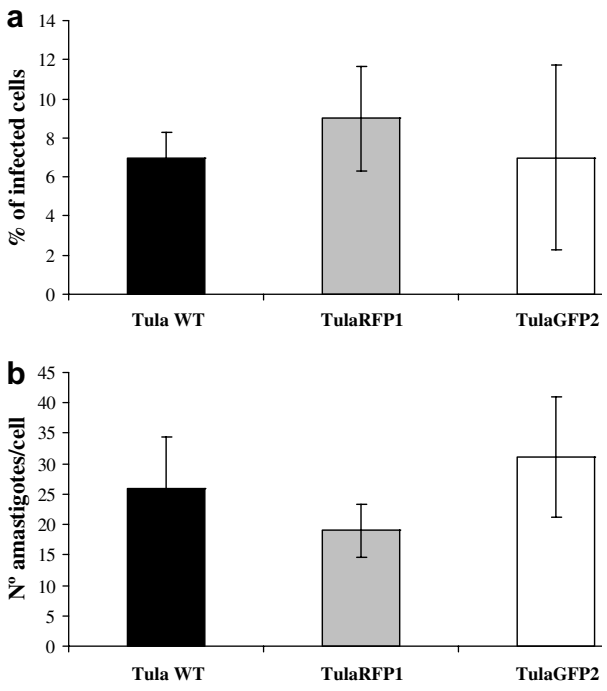

Fig. 3. Approximately, 10^5 Vero cells were platted and infected with 10^6 transfected trypomastigotes (TulaRFP1 and TulaGFP2 clones) or wild type parasites. After 6 days, cell monolayers were stained with Giemsa/May Grunwald. The percentage of infected cells (a) and the number of amastigotes per cell (b) were determined, with the numbers corresponding to the mean of three independent experiments.

ferences in the infectivity between transfected and WT parasites ($x^2 = 0.96$), suggesting that, at least for tissue culture cells, the transfection process did not compromise parasite infectivity.

The in vivo infectivity of fluorescent parasites was also evaluated using GKO mice which are highly susceptible to *T. cruzi* infection. This was necessary since preliminary studies using BALB/c mice, as well as other reports (Teixeira et al., 1999), have shown that inoculation of 100,000 trypomastigotes of either WT or GFP-expressing parasites from the Tulahuén strain resulted in extremely low levels of parasitemia. Two groups of five mice each were infected with WT or the TulaGFP2 clone. Twelve days after infection, we observed fluorescent *T. cruzi* in the blood drops from tails of all five mice infected with transgenic parasites (Fig. 4a and b). The intense fluorescence shown by the transfected parasites facilitated their identification and quantification in the animal bloodstream. When we analyzed other mouse tissues, fluorescent amastigotes were easily identified in the heart and diaphragm of animals infected with TulaGFP2 (Fig. 4c and d), whereas no parasites were found in the inflammatory foci of animals infected with WT trypomastigotes (data not shown). Therefore, although animals infected with the Tulahuén strain do not show intense parasitemia or tissue parasitism, the use of fluorescent *T. cruzi* clearly helps in the detection of parasites when using direct microscopic examination.

Finally, we incubated Vero cell cultures with TulaGFP1 and TulaRFP2 simultaneously. Four to 6 days later, we were able to detect single cells containing large numbers of both red and green amastigotes (Fig. 5a and b). Cells were also co-infected with transfected and cloned populations derived from two strains belonging to distinct *T. cruzi* lineages, a Colombiana strain expressing GFP (a *T. cruzi* I strain) and Tulahuén expressing RFP (a *T. cruzi* II strain) (Fig. 5c and d). The detection of green and red fluorescent amastigotes inside the cells, although rare (occurring in less than 1% of infected cells) clearly shows that a single host cell can be infected by parasites belonging to two distinct strains.

4. Discussion

Although several groups have reported the introduction of foreign genes in *T. cruzi*, few reports have described the use of integrative linear vectors containing sequences homologous to endogenous genes other than rRNA sequences to promote chromosomal integration of the exogenous gene. Most papers describe the use of vectors that replicate episomally, in the form of large concatemers, in transfected epimastigotes. In addition, these plasmids must be maintained in the cell under continuous drug selection. The introduction of sequences derived from the rRNA promoter, described by several authors (Teixeira et al., 1995; Tyler-Cross et al., 1995; Martinez-Calvillo et al., 1997; Vazquez and Levin, 1999; Dos Santos and Buck, 2000; Lorenzi et al., 2003), in addition to yielding a much greater expression of the reporter gene, facilitates the integration of the transfected vector into the nuclear genome by homologous recombination with the rDNA locus. Using the pTREX vector containing GFP and RFP genes to transfect *T. cruzi*, we have observed a high percentage of fluorescent cells as early as 12 h after electroporation. However, in contrast to other reports, the number of fluorescent parasites significantly diminishes 12 days after transfection even in the presence of drug selection (data not shown). Therefore, we designed a new vector that targets the integration of a foreign gene to the tubulin locus in the parasite genome, resulting in stable transfected parasite populations that can be cultivated even in the absence of drug selection (DaRocha et al., 2004).

Similar to the β-galactosidase-expressing *T. cruzi* described by Buckner et al. (1999), which was generated by integration of the *E. coli* lacZ gene into the calmodulin/ubiquitin locus, we targeted the integration of fluorescent GFP and RFP sequences into a multicopy gene array. After transfection, selection and cloning, the transgenic parasites showed high levels of green and red fluorescence even after 6 months of culture in the absence of drugs. The stability of this genetic modification allowed us to detect fluorescence not only in epimastigotes but also in trypomastigotes and amastigotes derived from mammalian cell cultures and in several tissues of infected mice. It is however intriguing that in all transfected cloned popula-

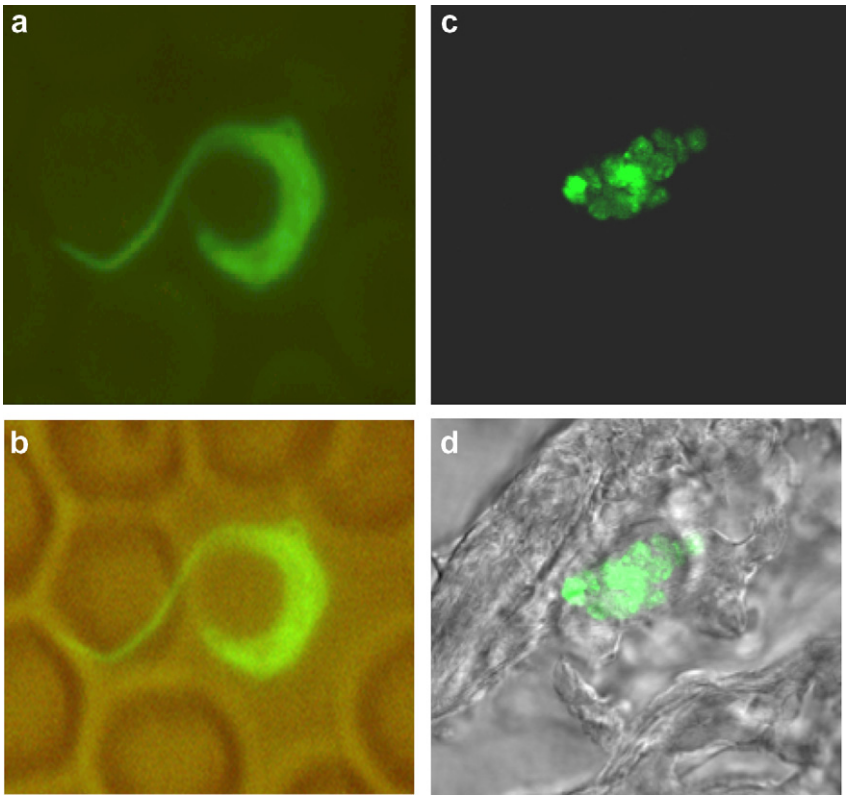

Fig. 4. Animal infection with tissue culture-derived trypomastigotes expressing green fluorescent protein (GFP). Fluorescent micrographs of mouse blood obtained 12 days p.i. of interferon-γ knockout mice containing a clone expressing GFP (TulaGFP2) at 100× magnification (a) and merged with a transmitted light image (b). (c and d) Confocal images of mouse heart tissue infected with the same clone (original magnifications 63×). (c) Confocal image for the GFP channel. (d) Merged differential interference contrast (DIC) image obtained with a Zeiss LSM 510 META confocal microscope.

tions we always observed a small amount of non-fluorescent cells even after several months of drug selection. GFP- and RFP-negative parasites continued to be observed even after one or more rounds of cloning procedures using a serial dilution protocol or plating in blood-agar media. Although we observed an increase in the percentage of green and red fluorescent cells after each step of the cloning procedure, none of the cultures displayed a population of parasites that was 100% fluorescent. As suggested by preliminary data analyzing G418-resistant parasite populations that have lost the fluorescent markers, the most likely explanation is the occurrence of additional recombination events that may take place after drug selection and cloning. Associated with regulatory mechanisms acting differentially in distinct genome locations, these recombination events may affect the expression of the GFP or RFP genes in a subset of the transfected cells.

Southern blot analyses indicated that GFP and RFP genes integrate at the tubulin locus in the genome of different *T. cruzi* strains. Here we have shown the integration of the RFP gene in the β-tubulin locus of the JG strain, which belongs to the *T. cruzi II* main lineage. In an earlier report, we showed that the same vector allowed integration of GFP in β-tubulin locus of the Col1.7G2 clone, a strain that belongs to the *T. cruzi* I lineage (DaRocha et al., 2004). Because tubulin sequences are highly conserved even among distant species, we anticipate that it is possible to generate GFP- and RFP-expressing parasites in most other *T. cruzi* strains.

To our knowledge, this is the first report describing the presence of stable, fluorescent parasites expressing GFP and RFP in infected animal tissues. Similar to the work described by Buckner and colleagues (1999), which used β-galactosidase staining to visualize the transfected parasites in host tissues, we used targeted integration of the foreign gene marker. Thus, we can speculate that plasmid integration into the parasite genome may contribute to selection of a more homogeneous transfected population, which facilitates the visualization of low numbers of amastigotes present in infected animal tissues. It must however be noted that with the Tulahuen strain, we were only able to detect GFP and RFP expressing parasites in GKO animals. Similarly, Santos et al. (2000) used GFP-expressing parasites that were transfected with an episomal vector to infect immunosuppressed mice. However, these authors were not able to show fluorescent parasites in infected animal tissues. Using the pTREX vector, Guevara et al. (2005) showed *T. cruzi* and *Trypanosoma rangeli* expressing GFP and RFP in the hemolymph of experimentally infected *Rhodnius prolixus*, in single and mixed infections. However, similar to the work by Santos et al. (2000), these authors did not show the presence of fluorescent parasites in animal

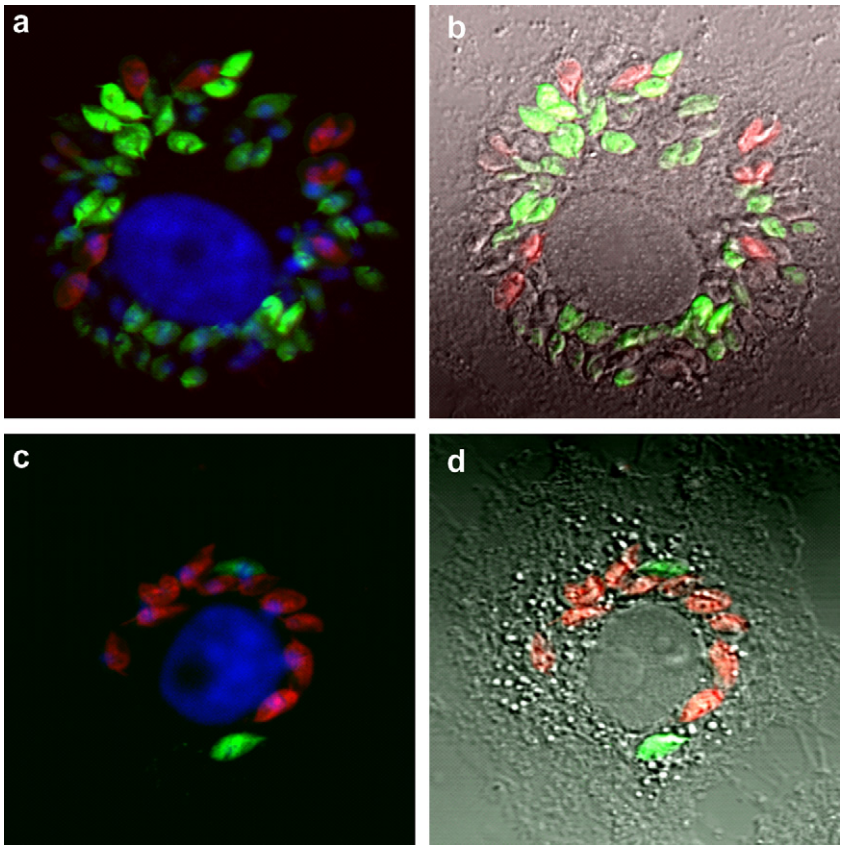

Fig. 5. Co-infection of Vero cells with parasites expressing different fluorescent proteins. Vero cell cultures were incubated with equal numbers of transfected trypomastigotes from the TulaRFP1 and TulaGFP2 clones. Four to 6 days after infection, cells were fixed, stained with DAPI and then intracellular fluorescent parasites were visualized by confocal microscopy using FITC and rhodamine filters to distinguish green and red amastigotes in the same infected cell (a and b). Vero cell cultures were also incubated with equal numbers of trypomastigotes from cloned transfected cell lines derived from the Colombiana (Col1.7G2GFP) and Tulahuén (Tula RFP1) strains (c and d). Cells were visualized at 80× original magnification using a Zeiss LSM 510 META confocal microscope. In b and d, merged fluorescence and DIC images are shown.

tissues. In contrast to transfected parasites derived from the Tulahuén strain, which required highly susceptible animals for infection, we were able to infect BALB/c mice with GFP expressing clones derived from JG, Colombiana and CL Brener strains. Although we were not able to visualize fluorescent parasites in several tissues including heart, diaphragm and rectum, probably because of the low parasitemia, infection of BALB/c mice with all of these strains allowed detection of fluorescent trypomastigotes in the bloodstream much easier than with WT parasites (not shown).

In one of our current studies involving the GFP- and RFP-expressing parasites, we incubated Vero cells with trypomastigotes expressing green and red fluorescence, to verify whether distinct parasites could replicate in the cytosol of the same cell. We demonstrated that, even when the parasites belong to two different *T. cruzi* lineages, they are able to infect the same cell. These fluorescent parasites are now being used to investigate the genetic exchange process in *T. cruzi*, a theme that has drawn much attention (Machado and Ayala, 2001; Pedroso et al., 2003; Gaunt et al., 2003; Freitas et al., 2006). In 2003, Gaunt et al. showed that two *T. cruzi* clones carrying two different drug-resistance markers and cultivated together through the entire life cycle generated double drug-resistant hybrids. These hybrids showed fusion of parental genotypes, loss of alleles, homologous recombination and uniparental inheritance of kinetoplast maxicircle DNA. Since hybrid clones were only detected after passage through mammalian cells, it has been speculated that the fusion event, which is thought to be very rare, occurs only during the mammalian amastigote stage. In contrast, using green and red fluorescent *Trypanosoma brucei*, (Gibson et al., 2006) have shown that genetic exchange occurs in the insect vector. The hybrid nature of yellow trypanosomes, present in the salivary glands, was confirmed by analysis of molecular karyotypes and microsatellite alleles. Although we have not detected any evidence for hybrids, the availability of *T. cruzi* expressing the two markers (GFP and RFP) will allow us to perform more in depth studies of genetic exchange in this organism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2007.08.013.

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