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***Leishmania (Viannia) panamensis*: An *in vitro* assay using the expression of GFP for screening of antileishmanial drug**

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Abstract

Promastigotes of *Leishmania (Viannia) panamensis* were successfully transfected with p6.5-*egfp* to express green fluorescent protein. The transfectants remained infective to macrophages, providing an *in vitro* model for screening antileishmanial drugs. This was demonstrated by flow cytometry of macrophage-associated GFP after exposure of infected cultures to known anti-leishmanial drugs, i. e. amphotericin B and glucantime®. Fluorescence of GFP diminished progressively from infected cells with increasing drug concentrations used in both cases. The availability of this fluorescent assay for infection of macrophages by *L. (V.) panamensis* facilitates drug discovery program for the *Viannia* species, which differ significantly from those of the *Leishmania* subgenus.

Index descriptor

L. (V.) panamensis; *L. (L.) amazonensis*; *L. (L.) major*; *L. (L.) donovani*; *Viannia* subgenus; *Leishmania* subgenus; leishmaniasis; green fluorescent protein; antileishmanial drug screening

Introduction

Leishmania spp. are responsible for leishmaniasis - a major parasitic disease of more than 12 million people in 88 countries. There are two million new cases annually, i. e. 1.5 million cutaneous cases and 500,000 visceral cases, respectively (WHO, 2004). Colombia is one of the major endemic places where 99% of the cases are cutaneous leishmaniasis and 70% are caused by *Leishmania (V.) panamensis*. Chemotherapy of this disease has relied mainly on pentavalent antimonials, but also on amphotericin B and pentamidine as the second line alternative drugs. Development of leishmanial resistance to antimonials (Rojas et al., 2006) necessitates prolonged treatments of patients with increasing dosages, resulting in severe side effects including death (Cesur et al., 2002; Oliveira et al., 2005). Recent evaluation of miltefosine yielded promising results (Soto and Soto, 2006), but the development of

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resistance to this drug in laboratory studies predicts its emergence in the clinics. There is thus always an urgent need to find new antileishmanial drugs.

Candidate compounds are usually screened against the amastigote as the clinically relevant stage by quantitative microscopy and/or by other assays for cell viability, e. g. propidium iodide permeability (Kamau et al., 2001) or radioactive biosynthetic labeling (Goto et al., 1995). Automation of such assays has been facilitated by transfection of *Leishmania* to express reporter genes encoding, for example, green fluorescent protein (GFP), luciferase and β -galactosidase that can be used for rapid and high-throughput drug screening. GFP of the jellyfish, *Aequorea victoria*, is often the marker of choice because it is non-toxic and auto-fluorescent for easy imaging by fluorescent microscopy and ready quantification by flow cytometry or fluorimetry. GFP also compares favorably for the simplicity of its use against other markers, i. e. luciferase, β -lactamase and β -galactosidase. Recently, GFP expressing transfectants have been developed from many species in the *Leishmania* subgenus, e. g. *L. (L.) infantum* (Kamau et al., 2001), *L. (L.) donovani* (Ha et al., 1996; Singh and Dube., 2004; Dube et al., 2005) and *L. (L.) amazonensis* (Chan et al., 2003; Okuno et al., 2003; Dutta et al., 2005). This is also true for other markers, e. g. luciferase in *L. (L.) donovani* (Roy et al., 2000; Gupta et al., 2005; Ashutosh et al., 2005), *L. (L.) infantum* (Serenio et al., 2001), *L. (L.) major* (Roy et al., 2000) and *L. (L.) amazonensis* (Lang et al., 2005), and β -lactamase and β -galactosidase in *L. (L.) amazonensis* (Buckner and Wilson, 2005; Okuno et al., 2003). In contrast, similar transfectants have not been made available for those in the *Viannia* subgenus, except two luciferase-transfected *L. (V.) panamensis* for drug studies (Romero et al., 2005; Henao et al., 2004).

Leishmania and *Viannia* are thought to have diverged 40 to 80 million years ago (McMahon-Pratt et al., 1992; McMahon-Pratt and Alexander, 2004). *Leishmania panamensis* of the *Viannia* subgenus differs significantly from its counterpart in the *Leishmania* subgenus, i. e. *L. (L.) major* in infectivity to laboratory animals (Goto et al., 1995; Guevara-Mendoza et al., 1997). Different species within the *Viannia* subgenus also vary among themselves in pathogenicity (Colmenares et al., 2002; McMahon-Pratt and Alexander, 2004), producing a spectrum of simple cutaneous, diffused cutaneous and mucocutaneous leishmaniasis. Intra-species heterogeneity is further noted in the *Viannia* subgenus (Cupolillo et al., 1997; Muskus et al., 1997; Pacheco et al., 2000; Saravia et al., 2002), including strain-specific drug sensitivity (Croft and Brun, 2003). Thus, additional models are needed for drug-screening to develop *Viannia*-specific chemotherapy.

In this study we report the development of GFP-transfectants from promastigotes of *L. (V.) panamensis*. These transfectants retain their infectivity to macrophage cell lines and continue to express GFP intracellularly *in vitro*. We demonstrated the utility of this infection model for potential high throughput screening of antileishmanial drugs *in vitro* by flow cytometry.

Materials and methods

Parasites and transfection

Promastigotes of *L. (V.) panamensis* (MHOM/87/CO/UA140), a Colombian isolate, was cultured at 25°C in Schneider's medium (Gibco) with 10% HIFBS in 25 cm² tissue culture flasks (Cellstar). For transfection, these cells grown to late-log phase were washed by centrifugation at 3,500 g at 4°C for 10 min once with PBS and twice in chilled electroporation buffer (25 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄ and 6 mM glucose). The parasite suspension (0.4 ml at 50 × 10⁶ cells/ml) was mixed with ~30 µg of p6.5-*egfp* (Dutta et al., 2005) in a 2 mm-gapped cuvette (BioRad) for electroporation in a Gene Pulser (BioRad) at 0.45 kV and 500 µF (Chen et al., 2000). After ~24 hrs of

incubation for recovery in drug-free medium with 20% HIFBS, the transfectants were selected for resistance to tunicamycin at 1.25, 2.5 and 5 $\mu\text{g/ml}$. Cells were microscopically monitored daily for viability and growth (Nikon Labophot-2).

Transfectants obtained were cloned by limiting dilution in 96-well tissue culture plates. Fluorescent clones emerged were picked and further selected with increasing concentrations of tunicamycin from 5 to 25 $\mu\text{g/ml}$. Transfectants grown at increasing selective pressures were harvest and fixed with 0.5% paraformaldehyde in PBS for flow cytometry (Beckman-coulter Epics XL) using an argon laser at 488 nm for excitation and 525 nm for emission. GFP-expressing promastigotes were analyzed flow cytometrically in 10,000 gated events and the numeric data were processed by using WinMDI software.

Infection of macrophages with GFP transfectants and determination of their median infective dose (IC_{50})

Monocyte-derived macrophages of the human cell line U937 were used to determine this after infection with GFP-transfectants (Sundstrom and Nilsson, 1976). The non-infected monocytes were cultured in RPMI 1640 medium (Sigma) plus 10% HIFBS at 37°C in 5% CO_2 and passaged every 2 days. These monocytes were treated at 3×10^5 cell/ml per well with 1 μM of phorbol myristate acetate (PMA) (Sigma) for 48 hr at 37°C in 24-well culture plates (Fisher). The macrophages so obtained were infected for two hrs at 34 °C with the GFP-transfectants at different parasite-to-host ratios. The cultures were washed three times with pre-warmed fresh medium to remove un-ingested parasites and further incubated for 24 hrs at 34°C. Cells were then removed from individual wells by vigorous aspiration with cold medium for reaction at 4 °C for 30 min with PE conjugated anti-CD33 (Pharmingen) (5 μl per sample) specific to the monocytic/myeloid lineage (Simmons and Seed, 1988). PE-conjugated isotype control was included to estimate the non-specific binding of the primary antibody to CD33. After washing, individual cell samples were subjected to two-color flow cytometric analysis, i. e. emission at 488 nm and excitation at 530 nm for GFP and 575 nm for PE. Aliquots of these samples at different parasite-to-macrophage ratios were simultaneously collected for microscopic evaluation of the percentage of infection after fixation in methanol and staining with 10% Giemsa (Merck). Median infective dose or IC_{50} was calculated from the data obtained by both methods using the probit method (Finney, 1971). Macrophages of the J774 line were also infected with the GFP-transfectants under conditions as described (Dutta et al., 2005).

Utility of the GFP transfectant-infected cultures for screening antileishmanial drugs by fluorescent assay

Two known antileishmanial drugs were included for comparison, i. e. meglumine antimoniate at 0.3125, 1.25, 5 and 20 $\mu\text{g/ml}$ (Glucantime®, Aventis Pharma Ltd, Brasil) and amphotericin B at 0.1563, 0.625, 2.5 and 10 $\mu\text{g/ml}$ (Bristol-Myers Squibb). Controls were diluents alone at the highest concentration used for each compound. The macrophages cultured in 24-well plates as described were infected for 2 hrs at 34 °C with the stationary phase GFP transfectants at the IC_{50} determined, i. e. a promastigote-to-macrophage ratio of 35:1. Cultures were washed to remove un-ingested promastigotes as before and then treated for 96 hrs at 34°C with the drugs at different concentrations as indicated. The drug effects were analyzed by flow cytometry of cell associated GFP and by microscopy as described. The EC_{50} was calculated from the fluorescence intensities of CD33 and GFP positive infected macrophages by using a logarithmic regression with the probit method (Finney, 1971).

Results

Expression of GFP in transfected *L. (V.) panamensis*

Fluorescent transfectants emerged after electroporation of cells under the initial selective pressures of 5 µg tunicamycin/ml, reaching a level of 50–60% of the total population by day 25. The proportion of GFP fluorescent cells increased significantly to 96% when clones were obtained and further selected for resistance to higher concentrations of tunicamycin from 10 to 25 µg/ml. The GFP fluorescence of individual cells appeared largely cytosolic and varied in intensity (Fig. 1A–D). Transfectants grown continuously with increasing selective pressures from 0 to 25 µg of tunicamycin/ml showed an increase in the fluorescent cells from 0 to 96% of the population (Fig. 2).

Assay for the activities of known antileishmanial drugs based on *Leishmania* GFP fluorescence intensities in infected macrophages

The transfectants retained their infectivity to the macrophages and therein differentiated into amastigotes, which remained fluorescent for the duration of the experiments, as seen by both fluorescent microscopy (Fig. 3) and flow cytometry (Fig. 4). GFP fluorescence was associated strictly with intracellular *Leishmania* in the parasitophorous vacuoles (Fig. 3A–D). Flow cytometry of the macrophages infected for 4 days showed that the percentage of GFP+ parasites increased in the CD33+ cells proportionally with increasing promastigote-to-macrophage ratios, indicative of increasing infection, as expected (Fig. 4A). The presence of CD33-negative cells (Fig. 4A, Lower left quadrant) suggests that a low percentage of PMA-treated U937 cells did not react with the anti-CD33-PE used. Estimation of the infection rate by the GFP fluorescence of the CD33+ cells is comparable to that obtained by microscopic enumeration of intracellular *Leishmania* in the transfectant-infected U937 cells, showing an increase with increasing promastigote-to-macrophage ratios from 19% to 74% by microscopy (data not shown) and from 17% to 58%, by flow cytometry. The median infective dose of the GFP transfectants is estimated to fall within the range of their number used at a parasite-to-macrophage ratio between 25:1 and 35:1.

Macrophages infected with the median infective dose of the GFP-transfectants were subsequently treated with meglumine antimoniate and amphotericin B for ~4 days at increasing drug concentrations mentioned. The EC₅₀s' of both antileishmanial drugs were determined by flow cytometry of GFP fluorescence levels in the CD33+ cells. The fluorescence intensities of GFP in arbitrary fluorescent unit (FU) decreased in these CD33+ populations gradually from 95.5 FU of the infected and untreated control to 48.17 and 41.03 FU, in those exposed to the highest concentrations of antimoniate and amphotericin B, respectively (data not shown). When these FU were converted to percentage of viable intracellular parasites, the viability decreased from 100% to 52% with antimoniate and from 100% to 0.8% with amphotericin B, in the infected cells exposed to the highest concentrations of these antileishmanial drugs (Fig. 4B). Namely, the CD33+GFP+ double fluorescent cells or infected macrophages decreased from 36.77% of the untreated control proportionally with increasing drug concentrations to 13.31% (Fig. 4C 1–3) and 7.61% (Fig. 4C 1, 4–5) for antimoniate and amphotericin B, respectively. The GFP+ CD33– populations were present in all cases, apparently representing amastigotes, which were inadvertently released from infected cells during their removal from the culture flasks by vigorous aspiration or as result of the infection itself. The proportion of CD33+ cells was reduced slightly with increasing concentration of both drugs, indicating that they are marginally toxic to U937 cells under the experimental conditions. However, the drug concentrations used were always below the LC₅₀. The EC₅₀s' of the antileishmanial compounds were determined to be 20 µg/ml and 0.078 µg/ml for meglumine antimoniate and Amphotericin B, respectively. These values are comparable, although not identical, to those previously

determined for wildtype cells by the conventional methods, e. g. an EC₅₀ value of 0.132 ± 0.09 µg/ml for amphotericin B (Unpublished).

Discussion

Genetic engineering of *Leishmania* to express fluorescent markers, such as GFP, facilitates drug screening automation. We produced such transfectants for *L. (V.) panamensis* with the p6.5-*egfp* constructs, which have been used previously only for species of the *Leishmania* subgenus (Chan et al., 2003; Dutta et al., 2005). Clonal variation in GFP expression was evident among the emerging transfectants in the initial selection. Stable clones were subsequently selected with increasing drug concentrations, giving intensive GFP fluorescence in almost 100% of the population (Figs 1 and 2). The vector p6.5 contains DNA sequences from a member of the *Leishmania* subgenus, *L. (L.) amazonensis*, including *nagt* as the selective marker for tunicamycin-resistance (Liu and Chang, 1992) and the precise deletion of a constitutively expressed gene (*p36*) 2.3 kb downstream of *nagt* as the expression site (Liu and Chang, 1994). Previously, p6.5 has been shown to express foreign genes with high yield in *L. (L.) amazonensis* (Sah et al., 2002) and other members of the *Leishmania* subgenus (Dutta et al, 2008). The work presented here represents first report for the utility of this *Leishmania* subgenus-derived vector for transfection of a member in the *Viannia* subgenus. Cross-subgenus functionality of the selective marker and the expression site in p6.5 is thus demonstrated. Whether this may contribute to the initial heterogeneity of GFP expression seen, is unknown. Inherent clonal variations of *L. (V.) panamensis* in gene expression and low selective pressures used initially are alternative possibilities, pending further investigation. The *egfp*-transfectants of *L. (V.) panamensis* retain not only its infectivity *in vitro* to human monocyte-derived macrophages of the cell line U937 and J774 macrophages of murine origin but also the ability to differentiate from promastigotes into amastigotes in these macrophages. More importantly, the intracellular parasites contain sufficient GFP fluorescence readily detectable by fluorescent microscopy (Fig. 3) and by flow cytometry (Figs. 4). There is a good correlation between parasite burdens and fluorescence quantitatively determined by microscopy and by flow cytometry. The latter method is less laborious, more accurate and higher in capacity to evaluate a large number of cells than possible by microscopy. This may be further simplified by fluorimetry of cell suspensions for quantitative measurement of GFP fluorescence.

Here, we also demonstrate the utility of *L. (V.) panamensis* transfected with GFP for evaluating their response to known antileishmanial compounds in the *in vitro* infection model described. The GFP-expression by the transfectants allows us to evaluate their infection of macrophages by measuring fluorescence, as shown previously for other intracellular pathogens, e. g. *Mycobacterium tuberculosis* (Collins et al., 1998), *Toxoplasma gondii* (Gubbels et al., 2004) and *Leishmania* subgenus species (Kamau et al., 2001; Singh and Dube, 2004; Dube et al., 2005). In our case, the stability of GFP fluorescence in the amastigotes of *L. (V.) panamensis* in infected cells makes it possible to show a dose-dependent antileishmanial activities of both glucantime® and amphotericin B. Their differential antileishmanial activities in favor of the latter drug are in agreement with previous reports (Escobar et al., 2001; Sereno et al., 2000).

In summary, data presented show that *L. (V.) panamensis* can be transfected to produce GFP in p6.5 expression vector, which has been shown to express foreign proteins efficiently in members of the *Leishmania* subgenus. This is the first report for such transfection of *L. (V.) panamensis* and its utility to evaluate antileishmanial compounds. The availability of these transfectants will facilitate the screening of drugs specific for the *Viannia* group and also the study of host-parasite interactions.

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Abbreviations

GFP	Green fluorescent protein
HIFBS	heat-inactivated fetal bovine serum
PMA	phorbol myristate acetate
PE	phycoerythrin
<i>nagt</i>	N-acetylglucosamine-1-phosphate transferase gene

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Figure 1. Fluorescent promastigotes of *L. (V.) panamensis* (UA-140) transfected with the p6.5-*egfp* constructs. (A and C), phase contrast; (B and D), fluorescent microscopy.

Figure 2.

Fluorescent cell population of GFP-expressing *L. (V.) panamensis* promastigotes increased with increasing selective pressure from 10–25 µg/ml of tunicamycin. Parasites used were grown for one cycle in drug-free medium before flow cytometry.

Figure 3.

GFP fluorescence of intracellular *L. (V.) panamensis* after infection of macrophages. (A and C), Phase contrast microscopy; (B and D), fluorescent microscopy. Macrophages of the J774 line were infected with *L. (V.) panamensis* transfectants as promastigotes at a parasite-to-macrophage ratio of 10:1 under the same conditions as described (Dutta et al., 2005). Culture medium was renewed daily for four days when samples were taken for imaging. U937 cells infected with these transfectants gave similar results.

Figure 4.

(A) Median infective dose (IC_{50}) of GFP-expressing *L. (V.) panamensis* in U937 cells. The IC_{50} was calculated from CD33 and GFP double-fluorescent cells in samples at increasing promastigote-to-macrophage ratios from 0:1 to 40:1 as indicated. [1] Isotype control: cells stained with a PE-conjugated mouse IgG1; [2] Non-infected cells stained with PE conjugated anti-CD33; [3–6] Infected cells using different parasite-to-macrophage ratio (5:1, 10:1, 20:1 and 40:1, respectively). (B) Determination of EC_{50} of two antileishmanial drugs for GFP-expressing *L. (V.) panamensis* in U937 cells. The EC_{50} was calculated from double emitting fluorescence cells for GFP and CD33 from a Dose-Response curve for meglumine antimoniate (upper graph) and amphotericin B (lower graph) by probit transformation. Curves show activities of antimoniate and amphotericin B at each concentration against intracellular amastigotes, as indicated by decreases in the percentage of fluorescence that is proportional to the number of parasites within cells when they are in presence of the corresponding drug concentration. (C) Infection rate of *L. (V.) panamensis* infected U-937 cells after treatment with increasing drug concentrations. Dot plots show leishmanicidal activities of meglumine antimoniate and amphotericin B each at the highest and the lowest concentrations against intracellular amastigotes, as indicated by decreases in the percentage of infection. [1] *L. (V.) panamensis* infected U-937 cells; [2–3] Infected cells after treatment with meglumine antimoniate at 0.3125 and 20 μ g/ml, respectively, and [4–5] Infected cells treated with amphotericin B at 0.156 and 10 μ g/ml, respectively.

Numerical values are specific cell population in % $\pm$ S.E. Left upper quadrant: CD33+, GFP–(uninfected U937 cells); right upper quadrant: CD33+, GFP+ (infected U937 cells); right lower quadrant: CD33–, GFP+ (extracellular parasites). Data presented were representatives of four independent experiments each performed in triplicate.