Applications of Recombinant *Leishmania amazonensis* Expressing egfp or the β -Galactosidase Gene for Drug Screening and Histopathological Analysis

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Abstract: *Leishmania amazonensis* recombinants expressing the enhanced green fluorescent protein (egfp) gene or β -galactosidase gene (lacZ) were constructed for drug screening and histopathological analysis. The egfp or lacZ in a leishmanial transfection vector, p6.5, was introduced into *L. amazonensis* promastigotes, and egfp or lacZ-carrying recombinant *L. amazonensis*, La/egfp and La/lacZ, respectively, were obtained. Expression of egfp or lacZ in both promastigotes and amastigotes could be clearly visualized by fluorescence microscopy or by light microscopy with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), respectively. Fluorescence signal and β -galactosidase activity measured by a colorimetric reaction with chlorophenol red β -D-galactopyranoside (CPRG) were well correlated to the numbers of these parasites. The inhibitory concentration (IC₅₀) of a leishmanicidal drug, amphotericin B, in *L. amazonensis* promastigotes measured using La/egfp and La/lacZ was similar to that measured by conventional methods such as cell counting, thymidine incorporation and colorimetric assay. Furthermore, the fluorescence signal and absorbance of CPRG correlated well with the numbers of La/egfp and La/lacZ amastigotes in macrophages, respectively, suggesting La/egfp and La/lacZ can be a convenient and useful tool for drug screening not only in promastigotes, but also in amastigotes of *L. amazonensis*. La/lacZ collected from mouse tissues four weeks after the parasite infection were stained well with X-Gal. La/lacZ allowed parasite detection at high sensitivity in the tissues of infected mice and will be useful for following infections in macrophages in vivo. Thus, the marker-transfected *Leishmania* parasites constructed in this study will be useful for analyses of *Leishmania* parasites, especially at the intracellular stage.

Key words: Recombinant *Leishmania amazonensis*, enhanced green fluorescent protein, β -galactosidase, drug screening, histopathological analysis

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Introduction

Protozoan parasites of the genus *Leishmania* are transmitted by the bite of sandflies, causing cutaneous, diffuse cutaneous, mucocutaneous and visceral leishmaniasis in humans [1, 5, 16]. The parasites alternate between two developmental stages, a flagellated extracellular promastigote, which lives in the midgut of the sandfly vector, and a non-flagellated intracellular amastigote, which resides in the phagolysosomes of vertebrate host macrophages.

The organic pentavalent antimonials, Pentostam and Glucantime, are currently the recommended first-choice drugs for the treatment of leishmaniasis. However, their strong side effects are a great problem [4]. Unfortunately, it has been reported that an acquired resistance to these drugs develops rapidly within endemic areas, particularly in response to drug pressure during a prolonged epidemic [9, 18]. The second-line of drugs, amphotericin B and pentamidine, remain of limited value because of toxicity and difficulty in administration [10]. New compounds with leishmanicidal activity are eagerly awaited.

The *in vitro* cultivation of promastigotes has facilitated assays for screening new leishmanicidal drugs. However, conventional methods, such as cell counting [7], the incorporation of radioactive nucleotides [14] or colorimetric assays [15, 21–23] have certain disadvantages, e.g. time consuming procedures, and the use of radioactive material. Many reports indicate that the *in vitro* activity of certain drugs against promastigotes differs greatly from that against intracellular amastigotes [24]. However, labor-intensive microscopic counting has been the only way to screen for compounds active against intracellular amastigotes [7]. Conventional methods for counting parasites cannot be applied to amastigotes perhaps, because of interference by host cell metabolism. Thus, more convenient methods, especially, for amastigote counting, are needed.

L. amazonensis is one of the most important parasites due to its wide spectrum of clinical manifestations [3, 5, 6]. The distribution of *L. amazonensis* in the early phase of infection should be studied, using mouse models, since it would indicate the fate of the disease [8]. Knowledge of tissue parasitism of *L. amazonensis* may contribute to a more logical approach to antiparasitic therapy. However, standard tissue-staining

techniques such as hematoxylin and eosin or Giemsa's staining do not permit easy visualization of tissue amastigotes. A better method for visualizing tissue parasites in animal models of *L. amazonensis* is needed.

Recent reports [2, 11, 19, 25] indicate that *egfp* and bacterial β -galactosidase can serve as markers for intracellular protozoan parasites. *Leishmania* parasites have neither *egfp* nor β -galactosidase gene. Mammalian cells emit a fluorescence which has a different peak in the excitation spectrum from *EGFP* employed in this study, and β -galactosidase which functions at a different pH from bacterial β -galactosidase [19], meaning that transfected *EGFP* and β -galactosidase activity in the recombinant parasites can be detected without any background derived from parasite and host cells.

In this study, recombinant parasites carrying the *egfp* and *lacZ* were constructed and used for drug screening and histopathological analysis.

Materials and Methods

Parasites

Promastigotes of *Leishmania amazonensis* strain MPRO/BR/72/M1845 were maintained at 25°C in 199 medium (NISSUI Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Trace Scientific LTD., Melbourne, Australia) and 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, ICN, Ohio, USA). Late log-phase parasites were harvested and used in the experiments [12].

Mice

Seven-week-old male BALB/c mice were purchased from SLC (Nippon SLC, Tokyo, Japan). After one week of acclimatization, they were used for the experiments. Mice were housed under specific pathogen-free conditions and utilized in accordance with institutional guidelines.

Amastigote culture

Peritoneal macrophages of BALB/c mice were applied onto glass coverslips (10⁶ macrophages per coverslip), placed in 24-well plates and incubated in RPMI 1640 medium (Invitrogen, California, USA) supplemented with 10% FCS at 37°C in 5% CO₂. After 24 h, macrophages were infected with parasites at a multiplicity of three parasites per cell and incubated at

34°C. After three hours, the cultures were rinsed in PBS (-) and incubated with fresh medium for 24 h.

Vector construction

The *EGFP*-coding region was removed from pCX-EGFP, which was provided by Dr. Jun-ichi Miyazaki (OSAKA University School of Medicine, Japan), and the fragment including the bacterial β -galactosidase-coding region was prepared from pCH110 (Clontech, California, USA). Both fragments were blunt-ended and inserted into the *Leishmania* gene expression vector, p6.5, at the *Bam*HI site in the sense orientation [13].

Transfection

L. amazonensis promastigotes were grown to the late log phase. Then, the cells were washed twice with PBS (-) and suspended in ice-cold HEPES-buffered saline (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄, and 6 mM glucose, pH 7.2) at a concentration of 1×10^8 cells/ml. Chilled p6.5 recombinants containing *egfp* or *lacZ*, respectively, were prepared in TE buffer (pH 7.4) at a concentration of 1 mg/ml. Cells were mixed with 5 μ g of plasmid in a 2 mm-gapped electroporation cuvette (BTX, San Diego, USA). The cells were electroporated with an Electro Cell Manipulator 600 (BTX) at 0.4 kV and 800 μ F. After incubation in fresh medium for 24 h, *egfp* and *lacZ*-transfected cells were selected with gradual acclimatization to tunicamycin (SIGMA, Tokyo, Japan) starting from a concentration of 5 μ g/ml [13]. The resulting transfectants carrying *egfp* and *lacZ* were designated *La/egfp* and *La/lacZ*, respectively.

Detection of enhanced green fluorescence protein (EGFP) by microscopic observation

La/egfp in promastigote forms was plated on slide glass and mounted with 50% glycerol-PBS (-) for fluorometric observation. For the study of amastigotes, peritoneal macrophages infected with *La/egfp* were prepared. The slides were mounted with 50% glycerol-PBS (-) for observation under a fluorescent microscope (BX50, Olympus, Tokyo, Japan).

Detection of β -galactosidase activity by microscopic observation

La/lacZ promastigotes were washed twice with PBS

(-) and fixed with 0.25% glutaraldehyde. Then, the cells were washed again and stained with X-Gal at 37°C for 16 h. The cells were rinsed in PBS (-) and counter-stained with Mayer's hematoxylin (Wako, Tokyo, Japan) at room temperature for 5 min. For the observation of amastigotes, peritoneal macrophages infected with *La/lacZ* were prepared. Then, the cultures were stained with X-Gal at 37°C for 4 h, counter-stained with Mayer's hematoxylin at room temperature for 5 min, and used for light microscopic observation under a BX50 fluorescent microscope.

Cell counting of promastigotes

Promastigotes of the wild-type, *La/egfp* and *La/lacZ* were seeded at 1×10^6 cells/ml with 199 medium in 25 cm² culture bottles. Then, parasite numbers were counted at 24-h intervals with a blood corpuscle counter (Erma, Tokyo, Japan).

Fluorometric assay of promastigotes

Fluorescence signals of *La/egfp* promastigotes cultured in 199 medium without phenol red (GIBCO BRL, New York, USA) in 96-well plates at 25°C were measured by a fluorescence microplate reader (Fluoro scan Ascent FL., Dainippon Pharmaceutical Co., Osaka, Japan) with excitation at 485 nm and emission at 538 nm. To determine the IC₅₀ of amphotericin B (ICN, Ohio, USA), *La/egfp* were cultured at 5×10^4 cells/ml with various concentrations of the drug, and their fluorescence signals were measured after 72 h incubation.

CPRG assay of promastigotes

La/lacZ promastigotes were cultured in 199 medium without phenol red in 96-well plates at 25°C. Before the measurement, CPRG (Roche Diagnostics, Tokyo, Japan) was added at a final concentration of 100 mM, and the culture plate was incubated for 3 h. CPRG cleavage was measured as absorbance at 570 nm with a reference of 630 nm by a Model 550 microplate reader (BIO-RAD, Tokyo, Japan). To determine the IC₅₀ of amphotericin B, *La/lacZ* were cultured in the same manner as *La/egfp*, and their absorbance was measured after 72-h incubation.

Alamer Blue assay of promastigotes

Wild-type parasites were plated as for the fluorometric assay. To each well, 20 μ l of Alamer Blue

(Bio-Source, California, USA) were added 3 h before the measurement and absorbance was measured after 72 h incubation by fluorescence microplate reader at an excitation of 544 nm and emission of 590 nm.

[³H]thymidine incorporation assay of promastigotes

Wild-type parasites were plated at 5×10^6 cells/ml in the same manner as for the fluorometric assay. One microCurie of [³H]thymidine (Amersham Pharmacia, Tokyo, Japan) was added to each well 6 h before the measurement. Cells were harvested onto glass-fiber filaments using a Skatron multiple automated sample harvester (Skatron, Sterling, USA) after 48 h incubation. Radioactivity was measured using a liquid scintillation counter (LSC-5100, Aloka, Tokyo, Japan).

Cell count of amastigotes

Peritoneal macrophages were infected with the wild-type, *La/egfp* and *La/lacZ* at multiples of 12, 6, 3, and 1.5 parasites per cell, as described above. The cultures were washed with PBS (-), fixed with 0.25% glutaraldehyde and stained with 0.1% nuclear fast red (Sigma, Tokyo, Japan) for cell counting. Infected parasites were enumerated by microscopic observation.

Fluorometric and CPRG assays of intracellular amastigotes

Macrophages infected with *La/egfp* and *La/lacZ* were prepared in RPMI 1640 medium without phenol red (GIBCO BRL, New York, USA). Their fluorescence signal and absorbance were measured as described above.

Infection

BALB/c mice were inoculated intracutaneously at the base of the tail (rump) with 1×10^7 cells/mouse of *La/lacZ* in a volume of 50 μ l of PBS (-) using a 25-gauge needle. The infected mice were sacrificed to collect tissue samples for recovering the parasite.

Parasite recovery

La/LacZ parasites recovered from skin lesions of BALB/c mice four weeks after inoculation were subjected to CPRG assay. Each lesion was cut into pieces and cultured with RPMI 1640 medium containing 25 μ g/ml of gentamycin at 25°C. After one week of incubation, recovered transfectants were transferred to 199

medium without tunicamycin for one week. The wild-type, *La/lacZ* and the recovered cells, designated *La/lacZ-le*, were plated at a concentration of 2×10^6 cells/ml in a final volume of 200 μ l in 96-well tissue culture plates, respectively, and the absorbance of CPRG was measured.

Histochemical staining

Stamp smears and frozen sections were prepared from the mice infected with *La/lacZ* by exsanguination under deep anesthesia at four weeks after the infection. Lesions were stamped on glass slides. Spleen was placed in 2% paraformaldehyde in PBS (-) with 2 mM MgCl₂ and fixed for an hour and rinsed twice with PBS (-). The tissues were transferred to 30% sucrose PBS 2 mM MgCl₂ and placed at 4°C for 16 h. Then, they were embedded in Tissue-tek O.T.C. compound (Sakura Finetechnical, Tokyo, Japan) and frozen at -80°C until use. Frozen sections of 20 μ m were cut by a cooled microtome (MICROM, Walldorf, Germany). The tissues were mounted on glass slides coated with APS (3-Aminopropyltriethoxysilane), and stored at -20°C. Frozen sections and stamp smears were used for X-Gal staining. The slides were scanned microscopically.

Statistical analyses

Student's *t*-test was used. Differences were considered significant for $P < 0.05$.

Results

Expression of egfp and lacZ in the transfected parasite

Expression of the transfected genes in *La/egfp* and *La/lacZ* in both promastigote and amastigote forms was demonstrated by fluorometric microscopic observation of *La/egfp* (Figs. 1A and 1B), and by light microscopic observation of X-Gal-stained *La/lacZ* parasites (Figs. 1C and 2D), respectively.

Growth of recombinant promastigotes measured by cell counting

The growth of *La/egfp* and *La/lacZ* was compared with that of wild-type parasites to check for any growth defects generated during transfection. The results indicate that there were no significant differences in growth between wild-type and recombinant parasites (Fig. 2).

Fig. 1. Expression of the transfected genes in *La/egfp* and *La/lacZ* both in promastigote and amastigote forms. Both recombinant parasites were cultured with tunicamycin. *Egfp* expression of *La/egfp* in both promastigote (A) and amastigote (B) forms was observed with oil immersion under a fluorescent microscope. β -galactosidase expression of *La/lacZ* in both promastigote (C) and amastigote (D) forms was observed with oil immersion under a light microscope. Bars indicate 5 μ m in each panel. N: nucleus. Arrowhead: intracellular amastigote.

Correlation of fluorescence signal/ β -galactosidase activity and the number of recombinant parasites

To examine the correlation between the amount of introduced gene product and the cell number, fluorescence signal/ β -galactosidase activity and number of transfected parasites were plotted. The data points of fluorescence signal vs. *La/egfp* parasite count (Fig. 3A) and of β -galactosidase activity vs. *La/lacZ* parasite count (Fig. 3B) showed linear relationships ($R^2 = 0.9927$ and 0.9827 , respectively).

Correlation of fluorescence intensity with parasite growth

La/egfp were cultured at 1×10^6 cells/ml and parasites were enumerated from days 1 to 5 (Fig. 4A). Then, parasite numbers and fluorescence signals of cultured *La/egfp* were plotted as shown in Figure 4B. Fluores-

Fig. 2. Growth curve of the wild-type, *La/egfp* and *La/lacZ* measured by cell counting. Growth curves of wild-type (■), *La/egfp* (◆) and *La/lacZ* (▲) are shown. The data points represent means of triplicate values with standard deviations.

cence signals increased as *La/egfp* promastigotes grew. A linear relationship was observed between fluorescence signals and parasite numbers from day 0 to 4 ($R^2 = 0.9979$).

Leishmanicidal activity of amphotericin B against *L. amazonensis* promastigotes

As an example of the application of recombinant parasites for drug screening, the leishmanicidal activity of amphotericin B against *L. amazonensis* promastigotes was measured with *La/egfp* and *La/lacZ*. The IC_{50} of amphotericin B for promastigotes was measured with *La/egfp* by fluorometric assay and with *La/lacZ* by CPRG assay. The IC_{50} of amphotericin B was also measured with wild-type promastigotes by Alamer Blue assay and [3H]thymidine incorporation assay. IC_{50} values were $0.42 \mu\text{g/ml}$ in the fluorometric assay (*La/egfp*), $0.71 \mu\text{g/ml}$ in the CPRG assay (*La/lacZ*), $0.71 \mu\text{g/ml}$ in the Alamer Blue assay and $0.24 \mu\text{g/ml}$ in the [3H]thymidine incorporation assay, respectively (Fig. 5).

Correlation of fluorescence intensity/ β -galactosidase activity and the number of amastigotes in macrophages

To examine the correlation between the amount of introduced gene product and cell number at the amastigote stage, numbers of amastigotes in cultured macrophages were compared with fluorescence signals and absorbance in the CPRG assay. At first, the growth *in vitro* of *La/egfp* and *La/lacZ* amastigotes was com-

Fig. 3. Correlation of fluorescence signal/absorbance and the number of transfected parasites. *La/egfp* and *La/lacZ* were serially diluted with 199 medium without phenol red and plated at the indicated concentrations. (A) Fluorescence signal of *La/egfp* was measured directly by a fluorescence microplate reader (excitation at 485 nm and emission at 538 nm). (B) *La/lacZ* was incubated with CPRG for three hours and the absorbance was measured at 570 nm with reference to 630 nm with a microplate reader. Linear relationships between the data points for fluorescence signal vs. the *La/egfp* parasite count and of β -galactosidase activity vs. the *La/lacZ* parasite count were observed ($R^2 = 0.9927$ and 0.9827 , respectively).

Fig. 4. Correlation between fluorescence signal and growth of *La/egfp*. (A) *La/egfp* was cultured and parasite numbers were scored by cell counting at 24 h intervals using a hemocytometer. (B) Fluorescence signals were measured simultaneously as described in materials and methods, and plotted with parasite number. Data at 0 h ($\bullet$), 24 h ($\circ$), 48 h ($\blacktriangle$), 72 h ($\triangle$) and 96 h ($\times$) are plotted. Values from day 0 to 4 were almost aligned ($R^2 = 0.9979$).

pared with that of wild-type amastigotes. *La/egfp* and *La/lacZ* showed similar levels of infectivity to the wild-type (Fig. 6A). The fluorescence signal of *La/egfp* and absorbance in the CPRG assay of *La/lacZ* in macrophages correlated well with the number of parasites determined by microscopic observation (Fig. 6B).

Detection of β -galactosidase activity in *La/lacZ* recovered from infected mice

La/lacZ promastigotes cultured without tunicamycin for four weeks were designated *La/lacZ* (-). The activity of β -galactosidase in *La/lacZ*-le and *La/lacZ* (-) measured by CPRG assay was similar but declined to one third of that of *La/lacZ* freshly isolated from the culture with 20 μ g/ml tunicamycin (Fig. 7). *La/lacZ*-le freshly isolated amastigotes in the stamp smears of in-

Fig. 5. Leishmanicidal activity of amphotericin B. The leishmanicidal activity of amphotericin B against *L. amazonensis* promastigotes was measured as fluorescence signal of *La/egfp* (A), as absorbance in the CPRG assay of *La/lacZ* (B), and by Alamer Blue assay (C) and [³H]thymidine incorporation assay (D) using wild-type parasite as described in materials and methods. The data points are means of triplicate values. The Y-axis indicates percent growth of the drug-treated parasites compared to those without drug-treatment.

Fig. 6. Infectivity of recombinant amastigotes and quantitation of induced gene products. The infectivity of the wild-type (■) *La/egfp* (□) and *La/lacZ* (□) in macrophages is shown (A). Each parasite was infected to peritoneal macrophages from BALB/c at a multiplicity of 12, 6, 3 and 1.5 per macrophage. Infected parasites were counted by oil immersion microscopic observation and the data were recorded as the parasite number per hundred macrophages. The data represent means of triplicate values with standard deviation. The correlation between number of infected *La/egfp* amastigotes and fluorescence signal (♦), and number of infected *La/lacZ* amastigotes and absorbance (●) is indicated (B). Linear relationships between the data points of fluorescence signal vs. *La/egfp* amastigote count and of β -galactosidase activity vs. *La/lacZ* amastigote count were observed ($R^2 = 0.976$ and 0.9652 , respectively).

Fig. 7. β -galactosidase activity in the wild-type, *La/lacZ* and *La/lacZ* (-). The latter was cultured *in vitro* without tunicamycin for four weeks. *La/lacZ-le* was recovered from lesions of mice four weeks after *La/lacZ* infection. The activity was measured as described in materials and methods (absorbance at 570 nm and reference at 630 nm). Data represent means of triplicate values with standard deviations.

infected mouse spleen (Fig. 8A) and cultured promastigotes from the isolated amastigotes (Fig. 8B) were well visualized by X-Gal staining with microscopic observation.

Detection of β -galactosidase-positive parasites in mouse tissue

Frozen sections were prepared from the spleen of BALB/c mice four weeks after *La/lacZ* infection, and stained with X-Gal. *La/lacZ* was well visualized and easily detected as blue spots (Fig. 9). Non-specific spots were not observed in the sections of wild-type-infected spleen (not shown).

Discussion

The *egfp* and *lacZ*-transfected *L. amazonensis*, *La/egfp* and *La/lacZ*, were obtained. Judging from the growth of the recombinant parasite compared with that of the wild-type (Fig. 2), the *egfp* and *lacZ* expression did not have any serious effect on the biological activity of the transfected cells, although this has been questioned earlier [17].

Figure 3 shows the good correlation of the fluorescence signals of the fluorometric assay and absorbance of the CPRG assay to the parasite numbers of *La/egfp* and *La/lacZ*, respectively. This means that the fluores-

Fig. 8. X-Gal staining of *La/lacZ-le*. Stamp smear of spleen of a *La/lacZ*-infected mouse (A) and cultured promastigotes isolated from the lesion of an infected mouse (B) were stained with X-Gal and observed with oil immersion under a light microscope. Bars indicate 5 μ m in each panel. N: nucleus. Arrowhead: intracellular amastigote.

Fig. 9. Detection of *La/lacZ* in the spleen of infected mice by histochemical staining with X-Gal. Frozen sections of spleen collected from BALB/c mice after four weeks of *La/lacZ* infection were prepared and stained with X-Gal as described in materials and methods. Sections were observed with oil immersion under a light microscope. Arrowhead indicates representatives of X-Gal-stained *La/lacZ*. Bar indicates 10 μ m.

cence signals of *La/egfp* and absorbance of *La/lacZ* directly reflected the number of parasites. As shown in Fig. 4, fluorescence signals were closely related to parasite numbers. The transfectants were available for the quantitative analysis of promastigotes, such as drug screening, at least up to 72 h of incubation in the system employed in this study. Notably, the fluorometric assay using *La/egfp* does not require additives and mea-

surements can be made directly.

Amphotericin B, known as an effective leishmanicidal drug [10], was employed as a test agent for the evaluation of a drug screening method using *La/egfp* and *La/lacZ*. The IC_{50} of amphotericin B for *L. amazonensis* promastigotes measured with *La/egfp* and *La/lacZ* was very similar to the results obtained by the Alamer Blue assay and [3H]thymidine incorporation assay in this study (Fig. 5). It confirmed that both transfectants could be used for drug screening in promastigotes. However, many drugs undergo absorbance and this can interfere with the results. More than one assay system, such as the fluorometric assay of *La/egfp* and the CPRG assay with *La/lacZ*, should be prepared in such cases.

Since many reports indicate that the *in vitro* activity of certain drugs against promastigotes is greatly different from that against intracellular amastigotes [7, 23], drug screening should be performed by using amastigote-stage parasites. However, the quantitation of intracellular parasites, amastigotes, requires hours of labor-intensive counting by microscopy. Conventional colorimetric assays, such as the Alamer Blue and MTT assays, may not be applicable, since the metabolism of the host macrophage would interfere with the results, and a convenient method to measure the leishmanicidal effects of new compounds on intracellular parasites is awaited.

As shown in Fig. 6A, there was no significant difference in the infectivity of the wild-type and recombinant amastigotes. When *La/egfp* and *La/lacZ* were introduced into BALB/c mice, lesions formed, suggesting that the expression of *egfp* and *lacZ* had little influence on the virulence of the parasite *in vitro* (data not shown). Since the fluorescence signals and β -galactosidase activity of the parasite-infected cultures reflected the number of infected amastigotes (Fig. 6B), *La/egfp* and *La/lacZ* can also be applied to the screening of drugs in *Leishmania* amastigotes.

An anticipated problem in drug screening using *La/egfp* and *La/lacZ* is the release of active EGFP or β -galactosidase from lysed intracellular parasites by macrophages resulting in a false positive readout for parasite growth. The half-life of released enzymes should be analyzed.

Figure 7 shows β -galactosidase activity in *La/lacZ*-le collected from lesions on the rump in BALB/c mice after four weeks of infection. Although the activity of

β -galactosidase in *La/lacZ*-le declined compared to that of *La/lacZ*, it was well maintained even in the absence of drug pressure for at least four weeks *in vivo* (Fig. 7). Since the β -galactosidase activity of *La/lacZ* (-), which had been cultured for 4 weeks without tunicamycin, also declined to the same degree as that of *La/lacZ*-le, the decrease in the β -galactosidase activity of *La/lacZ*-le was not due to immunological or other pressures *in vivo*. The β -galactosidase activity of *La/lacZ* increased as the tunicamycin concentration in the culture was raised, maybe due to the increase in the copy number of the p6.5 plasmid (data not shown). The reduction in activity, therefore, would be due to the decrease in the copy number of the transfected plasmid, since p6.5 episomal vector [13] was used in the present study. However, all *La/lacZ*-le parasites were still positive for the expression of β -galactosidase and were easily detected by X-Gal staining under the microscope (Fig. 8). X-Gal-stained parasites were clearly visible as blue spots, even in the thick frozen sections (20 μ m thickness), suggesting that the X-Gal staining method described in this paper has an advantage in terms of sensitivity over standard-tissue staining methods, such as hematoxylin and eosin or Giemsa's staining, using paraffin sections (3 μ m thickness) or stamp smears (small number of cells) for parasite detection.

As for further improvement, it may be better for the *egfp* and β -galactosidase genes to be integrated into the leishmania genome for the stable expression of the *egfp* and *lacZ*, as reported by Miblit et al. [20].

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References

1. Aldina, B., Diana, P.S., Gabriel, G.Jr., Hooman, M., Diane, M.P., Amelia, R.J., Roque, A., Robert, B., Manoel, B.N.,

- Edgar, M.C., and Warren, D.J.Jr. 1991. Leishmaniasis in Bahia, Brazil: Evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease. *Am. J. Trop. Med. Hyg.* 44: 536–546.
2. Abdullah, S.M., Flath, B., and Presber, W. 1998. Mixed infection of human U-973 cells by two different species of *Leishmania*. *Am. J. Trop. Med. Hyg.* 59: 182–188.
 3. Almeida, R.P., Barral-N. M., Jesus, A.M.R., Frietas, L.A.R., Carvalho, E.M., and Barral, A. 1996. Biological behavior of *Leishmania amazonensis* isolated from human with cutaneous, mucosal, or visceral leishmaniasis in BALB/c mice. *Am. J. Trop. Med. Hyg.* 54: 178–184.
 4. Balana-Fouce, R., Reguera, R.M., Cubria, J.C., and Ordonez, D. 1998. Review: The pharmacology of Leishmaniasis. *Gen. Pharmac.* 30: 435–443.
 5. Barrel, A., Badaro, R., Diana, Barral, N.M., Grimaldi, Jr.G., Momen, H., and Calvalho, E.M. 1986. Isolation of *Leishmania mexicana amazonensis* from the bone marrow in a case of American Visceral Leishmaniasis. *Am. J. Trop. Med. Hyg.* 35: 732–734.
 6. Barral, A. 1991. *Leishmania amazonensis* is implicated in the whole spectrum of human clinical leishmaniasis. *Am. J. Trop. Med. Hyg.* 44: 536–546.
 7. Berman, J.D. and Lee, L.S. 1984. Activity of antileishmanial agents against amastigotes in human monocyte-derived macrophages and in mouse peritoneal macrophages. *J. Parasit.* 70: 220–225.
 8. Blackwell, J., Freeman, J., and Bradley, D. 1980. Influence of H-2 complex on acquired resistance to *Leishmania donovani* infection in mice. *Nature.* 283: 72–74.
 9. Borst, P. and Ouellette, M. 1995. New mechanism of drug resistance in parasitic protozoa. *Annu. Rev. Microbiol.* 49: 427–460.
 10. Brajtburg, J. and Bolard, J. 1996. Carrier effects on biological activity of Amphotericin B. *Clin. Microbiol. Rev.* 9: 512–531.
 11. Buckner, F.S., Wilson, A.J., and Voorhis, W.C. 1999. Detection of live *Trypanosoma cruzi* in tissues of infected mice by using histochemical stain for β -galactosidase. *Am. Soc. microbiol.* 403–409.
 12. Detke, S., Chaudhuri, G., Kink, J.A., and Chang, K.P. 1988. DNA amplification in tunicamycin-resistant *Leishmania Mexicana*. Multicopies of a single 63-kilobase supercoiled molecule and their expression. *J. Biol. Chem.* 263: 3418–3424.
 13. Chen, D.Q., Kolli, B.K., Yadaba, N., Lu, H.G., Gilman-Sachs, A., Peterson, D.A., and Chang, K.P. 2000. Episomal expression of specific sense and antisense mRNAs in *Leishmania amazonensis*: modulation of gp63 level in promastigotes and their infection of macrophages *in vitro*. *Infect. Immun.* 68: 80–86.
 14. Desjardins, R.E., Casero, R.A.Jr., Willet, G.P., Childs, G.E., and Canfield, C.J. 1980. *Trypanosoma rhodesiense*: Semiautomated microtesting for quantitation of antitrypanosomal activity *in vitro*. *Exp. Parasitol.* 50: 260–271.
 15. Ellis, J.A., Fish, W.R., Sileghem, M., and McOdimba, F. 1993. A colorimetric assay for trypanosome viability and metabolic function. *Vet. Parasitol.* 50: 143–149.
 16. Grimerdi, Jr.G., Tesh, R.B., and McMahon, P.D. 1989. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. *Am. J. Trop. Med. Hyg.* 41: 687–725.
 17. Hsiao, S.L., Ming, S.J., Chao, K.C., Ping, H.C., and Nir, J.K. 1999. Is green fluorescent protein toxic to the living cells? *Biochem. Biophys. Res. Commun.* 260: 712–717.
 18. Max, G. Tina, N.T., and Eileen, D.F. 1992. Drug resistance in Leishmaniasis: Its implication in systemic chemotherapy of cutaneous and mucocutaneous disease. *Am. J. Trop. Med. Hyg.* 47: 117–126.
 19. McFadden, D., Seeber, F., and Boothroid, J.C. 1997. Use of *Toxoplasma gondii* expressing β -Galactosidase for colorimetric assessment of drug activity *in vitro*. *Antimicrob Agents and Chemother.* 1849–1853.
 20. Miblit, A., Motram, J.C., Overath, P., and Abischer, T. 2000. Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in *Leishmania* amastigotes. *Mol. Biochem. Parasitol.* 107: 251–261.
 21. Mikus, J. and Steverding, D. 2000. A simple colorimetric method to screen drug cytotoxicity against *Leishmania* using the dye Alamer Blue. *Parasitol Inter.* 48: 265–269.
 22. Obexer, W., Schmid, G., and Brun R. 1995. A novel *in vitro* screening assay for trypanocidal activity using the fluorescent dye BCECF-AM. *Trop. Med. Parasitol.* 46: 45–48.
 23. Sereno, D. and Lemesre, J.L. 1997. Use of an enzymatic micromethod to quantify amastigote stage of *Leishmania amazonensis in vitro*. *Parasitol. Res.* 83: 401–403.
 24. Simon, L.C. 1988. Recent developments in the chemotherapy of leishmaniasis. *TIPS-October* 9: 376–381.
 25. Valdivia, R.H., Hromockyj, A.E., Monack, D., Ramakrishnan, L., and Falkow, S. 1996. Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. *Gene.* 173: 47–52.