REAL TIME IN VIVO GFP IMAGING IN A MURINE LEISHMANIASIS MODEL: A NEW TOOL FOR LEISHMANIA VACCINE AND DRUG DISCOVERY

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**ABSTRACT:** *Leishmania* are obligate intracellular protozoan parasites that cause a broad spectrum of clinical diseases in mammalian hosts. The most frequently used approach to quantify parasites in murine model systems is based on thickness measurements of the footpad or ear after experimental infection. To overcome the limitations of this method, we used a *Leishmania* mutant, episomally transfected with enhanced green fluorescent protein (GFP), enabling *in vivo* real-time whole-body fluorescence imaging to follow the progression of *Leishmania* infection in parasitized tissues. Fluorescence correlated with the number of *Leishmania* in the tissue, and demonstrated the real time efficacy of a therapeutic vaccine. This approach provides several substantial advantages over currently available animal model systems for the *in vivo* study of immunopathogenesis, prevention, and therapy of leishmaniasis. These include improvements in sensitivity and the ability to acquire real-time data on progression and spread of the infection.

**Introduction**

*Leishmania* have been known human pathogens for centuries and continue to cause significant morbidity and mortality. Over 350 million people are at risk of *Leishmania* infection and at least 500,000 new cases with severe morbidity are reported yearly. Additionally, *Leishmania* spp. are emerging as important opportunistic pathogens in persons co-infected with HIV-1. Therapeutic options for managing leishmaniasis are limited, and many have significant toxicity. Rapid advances in computational proteomics, and synthetic chemistry coupled with new high throughput screening technologies raise the likelihood that within a few years many new compounds...
could be ready for testing in animal model systems. Furthermore, several groups around
the world are exploring novel and previously characterized molecules for use as vaccines
against leishmaniasis. (6,14)

Murine models of leishmaniasis have been extensively used to study the
pathogenesis of the disease and to test novel therapeutic and immunoprophylactic
agents. (18) *Leishmania* infection of BALB/c mice often results in uncontrolled growth
of the parasite at the primary site of the infection followed by progressive dissemination
through the lymph nodes into the reticulo-endothelial system. (22) These mice are
susceptible to infection by various *Leishmania* species, including *L. major* and *L.*
*amazonensis* presented here. (7,17) The standard method for following infection in this *in
vivo* model is based on estimation of parasite loads by labor intensive microscopic
enumeration of sacrificed animals, or caliper based measurements of the size or thickness
of lesions developed at the site of infection such as the footpad, ear dermis or tailbase.
(7,22)

Optical techniques are now available for whole body imaging of small animals
using luciferase and green fluorescent protein (GFP). (10,11,25,26) Bioluminescent
*Leishmania* expressing luciferase has recently been used for monitoring infection in
mouse macrophages, as well as in living mice. (15) Several of us have pioneered *in vivo*
imaging with green fluorescent protein to visualize tumors and infectious agents such as
*Salmonella typhimurium* in live mice. (10,25,27) Recently, in an experimental model of
malaria, imaging of *Plasmodium berghei* transfected with GFP demonstrated the
dynamics of infection directly in live mice. (1) GFP transfected *Leishmania* have been
used to screen for anti-leishmanial activity in cell cultures by flow cytometry and
microtiter plate assays. Herein we describe the use of episomally GFP transfected *Leishmania* in a whole body imaging system to follow the dynamics of infection.

**Materials and Methods**

This study was conducted under UCSD animal protocol #S05322 under the auspices of the UCSD Institutional Animal Care and Use Committee.

**Transfected Parasites**

*egfp*-transfectants of *L. amazonensis* (LV78, clone 12-1) were prepared as described. Briefly, *egfp* was cloned into the BamH1 site of p6.5, a *Leishmania*-specific vector, for expansion in *E. coli* and the constructs obtained were used to transfect stationary phase promastigotes by electroporation. Electroporated cells were then selected for resistance to tunicamycin at 10 µg/ml, and stable transfectants were passaged continuously under these selective conditions *in vitro* in Medium M199 buffered to pH 7.4 with 50 mM HEPES and fortified with 10% heat-inactivated fetal bovine serum.

**In vitro culture system**

*egfp*-transfected *Leishmania amazonensis* parasites harvested from mouse footpads were expanded in standard non-ventilated tissue culture flasks in M199 media supplemented with 20% heat-inactivated FCS, 1 mg Hemin (Sigma H-1652), 0.25 ml of 0.1% Biotin in 95% EtOH (Sigma B-4639), 50mM HEPES (Sigma H-1552), 100 U/ml penicillin, and 100 µg/ml streptomycin (GibcoBRL 25030-081). Cultures were maintained at 25 °C, and the parasites passaged by 1:9 dilution weekly. The transfectants
were grown for one cycle without selective pressure before inoculation so as to avoid the introduction of cytotoxic tunicamycin into the recipient mice.

In vivo (murine) model of infection

The right hind footpads of female BALB/c mice aged 8-12 weeks were subcutaneously injected each with $10^7$ egfp\textsuperscript{-transfected} late-stationary phase *L. amazonensis* promastigotes suspended in 100 \( \mu \text{l} \) of phosphate buffered saline (PBS), using a 26 gauge needle. GFP fluorescence was demonstrated within mouse footpads up to 3 months after inoculation (unpublished observations).

Imaging

The footpads of the mice were imaged weekly beginning at day 0 with the Olympus OV-100 whole-mouse imaging system as described below.\(^{(26)}\) Mice were treated with a depilatory substance (Nair\textsuperscript{®}) to remove hair from their legs and feet to reduce background autofluorescence. They were then temporarily anesthetized with xylazine/ketamine/acepromazine solution given intraperitoneally, and then imaged on days 0, 7, 14, 24, 28, and 35 after inoculation. Photographs were taken after exposure for 1.5 seconds with a focal length of 40.7 mm. Pixel counting and measurement of the lesions were performed using Olympus\textsuperscript{®} software. Measurements were reported as “sum green”, a quantitative measurement defined as the number of green pixels in a given area multiplied by the average intensity of each pixel.

Presence of *Leishmania* within tissue

Mice were sacrificed for collecting samples from infected footpads, from which frozen sections of 5 microns in thickness were prepared. Fluorescence pictures were taken using a Nikon E600 microscope prior to fixation. Additional sections were cut and
fixed with cold acetone and stained with F4 80 (rat anti-mouse macrophage antibody subsequently biotinylated and labeled with phycoerythrin) and DAPI, to demonstrate co-localization.

Immunotherapy with Leish 11f+ MPL-SE® vaccine

BALB/c mice divided into groups of five were inoculated in the right footpad with $10^7$ egfp transfected *L. amazonensis* promastigotes. On days 7, 14 and 24 the mice in the control arm received subcutaneous injections 25 µl of PBS into the footpad. The mice in the vaccine group received 100 µl of the Leish 11f + MPL-SE® vaccine on days 7, 14 and 24 of which 30 µl was injected subcutaneously into the inoculated footpad, and 70 µl injected subcutaneously into the right flank of the mouse. Imaging was performed weekly as described above, and the mice were euthanized on day 41 of the experiment.

Statistical Analysis

Prism 4.0® (Graphpad Software, Carlsbad, CA) was used to create charts and best curve fits using a non-linear regression exponential growth model. Intergroup comparisons were analyzed by the unpaired Students T-test using the same statistical package.

Results

In order to determine whether we could detect GFP labeled transfectants *in vivo* using whole body imaging, we subcutaneously injected $10^7$ GFP transfectants into the hind footpads BALB/c mice. (9,13) The mice were examined weekly for five weeks with the Olympus OV-100® (Olympus, Japan) fluorescence small animal imaging system.(25) GFP fluorescence, which was initially localized to the site of the inoculation,
subsequently spread progressively to a wider area over the course of the next five weeks (Figure 1). Progression of the lesion with time of infection was easily and precisely delineated by measuring pixel counts of the fluorescent images using the Olympus OV-100® imaging software. Measurements towards the end of the five week period showed increasing thickness of the footpads. However, the first several measurements for each of the mice were variable and did not demonstrate a clear trend of increasing thickness.

In Figure 2, BALB/c mice were inoculated with increasing numbers of egfp transfected *L. amazonensis*. The time of initial detection using the in vivo imaging method is plotted for each of the inoculums placed. By our estimation, until there was a 40% increase in footpad size, the variability in the measurements precluded any determination of whether or not true infection is present. The relationship of footpad thickness with fluorescence shows that it took 12 more days after initial detection by imaging (Figure 1G) before we felt that we could adequately deduce infection using the traditional caliper based method.

The fluorescence visualized by whole body imaging was confirmed to be *Leishmania* by direct fluorescence microscopy of infected tissues. *Leishmania* amastigotes were seen as fluorescent bodies in five micron cryo-sections (Figure 3a). Persistence of fluorescence 5 weeks after inoculation was demonstrated in individual parasites in Figures 3b - 3d. Immunostaining of the preparations demonstrated co-localization of the GFP with macrophages verifying that the *Leishmania* amastigotes were intracellular (not shown).

GFP fluorescence intensity was reflective of parasite burden in the footpads, as demonstrated by the footpads of seven mice that were inoculated with increasing
concentrations of egfp transfected Leishmania amazonensis, and then imaged. We found that the sum green pixel counts from the imaging studies were proportional to the number of the transfectants injected into each footpad (Figure 4), suggesting that imaging could be used as a semiquantitative correlate of the number of parasites in vivo.

We then applied this approach to the evaluation of an experimental immunotherapy against leishmaniasis that had previously been shown to be clinically effective on a compassionate use basis. Recently, the use of the recombinant polyprotein Leish 111f + MPL-SE®, comprised of the leishmanial antigens in equal parts designated as TSA (heat shock protein 83), LmSTI1 (stress inducible protein 1) and LeIF (Leishmania elongation initiation factor), and MPL-SE® as an immunological adjuvant, demonstrated efficacy in the treatment of refractory mucosal leishmaniasis.(2) We inoculated two groups of five mice each with $10^7$ GFP transfected promastigotes from culture into mouse footpads. Infection became visible by tissue imaging for GFP fluorescence one week after inoculation. Swelling of inoculated footpads was not demonstrable using traditional caliper-based methods until almost 3 weeks after the inoculation. We began weekly immunotherapeutic treatments one week after inoculation with the Leish 111f +MPL-SE® vaccine and PBS alone for the control group. Progression of the infection was monitored by real-time imaging for GFP intensity. Immunotherapy of the infected mice for three weeks with the vaccine significantly suppressed the infection when compared with the control. This was shown much earlier using measurement of GFP intensity when compared to footpad measurements (Figure 5). The results obtained are consistent with recent work by Calvopina et al. who investigated the therapeutic effects of soluble Leishmania amastigote antigens in
combination with the synthetic lipid-A analog ONO-4007 as an adjuvant in a murine model of cutaneous leishmaniasis as assessed by footpad swelling.(4)

Discussion

Our work describes the application of whole-body fluorescent imaging in the murine model for the study of leishmaniasis. This new method using GFP transfected *Leishmania* to induce murine cutaneous leishmaniasis is a novel dynamic immunopathogenic model that allows visualization and correlation of fluorescent intensity with parasite burden. Our fluorescence measurements correlate with parasite burden and are more sensitive and precise than the standard caliper based method of following *Leishmania* infection *in vivo*. Although the sensitivity of detection was $10^6$ microorganisms in this model, previous experiments established that at this level of microorganism burden, the size of the lesion was undetectable by caliper measurements but clearly visible by fluorescent imaging.

Luciferase has also been used as a reporter for *in vivo* imaging of *Leishmania* and *Leishmania* stably transfected with luciferase have been detected *in vivo* using a ear model one day after $10^5$ organisms were inoculated.(15) However, there are several limitations to this approach. Luciferase requires the substrate luciferin, which must be administered intravenously or intraperitoneally each time imaging is performed. The luminescence produced is unstable, and dependent upon the metabolic activity of cells transfected with luciferase which can vary depending upon where in the lesion parasites are located, time of day, and other factors. Finally, fluorescence imaging allows for actual imaging, while quantification with luminescence requires photon counting, and can only generate a
pseudo image. This is important when studying tissue harvested from an infected animal, as parasites can be localized individually.

Fluorescent imaging offers several advantages. *In vitro* systems have demonstrated that fluorescence measurements are proportional to the number of *Leishmania* amastigotes present.(20) Episomal transfection was chosen because it provided significantly higher levels of fluorescence than when Roy *et al.* demonstrated successful chromosomal integration of the luciferase gene into the ribosomal promoter region of *L. major* and *L. donovani*. In their work they noted that these parasites were almost 2 log$_{10}$ less luminescent than the episomal transfectants.(21) Recent work has shown that *Leishmania* constitutively express their entire genome, and that gene expression modification occurs post transcriptionally.(16) Because of this, the expression of a gene integrated into the Leishmania chromosome will be dependent on many factors. *Leishmania* transfectants retain episomal plasmids in the absence of selective pressure for prolonged periods.\(^{23}\) GFP expression stability *in vivo* has been well demonstrated previously in tumor growth, metastasis, and angiogenesis models.(10) In hamster ovary cells, episomal expression of GFP has been shown to remain stable even after 24 days in the absence of selective pressure.(19) Furthermore, in our system we followed mice infected with 100 GFP transfected *Leishmania* for greater than eight weeks and despite the lack of selective pressure *in vivo*, fluorescence was visualized after 70 days. Previous studies that have classically described footpad measurements using calipers, estimate the severity of infection by measuring the thickness of the footpad, observing for signs of ulceration, and monitoring other clinical parameters that do not necessarily represent the burden of infection but may reflect inflammation. (3,6) In our
model we directly measure parasite burden using the fluorescence expressed by the parasites. Our fluorescence imaging system gives a precise two dimensional image of the extent of infection, independent of the inflammatory response. This measurement method has been well established in the measurement of fluorescing tumor lesions in vivo, and we now describe its use in murine cutaneous leishmaniasis. (25) The tissue penetration of the GFP fluorescence and our imaging software allow us to make an approximation of the integration of total fluorescence through multiple planes. Therefore, although we are using single plane imaging, we can determine an approximation of the total volume of infection. The future may yield improvements in small animal imaging resolution through tomography or volumetric imaging using Z-series, a method of imaging that allows a more 3 dimensional view of the image by taking images in serial depths similar to tomography.

The sensitivity of this technique makes it extremely useful for following Leishmania in vivo. The intensity of GFP fluorescence and the sensitivity of our detection system gave us a limit of detection of ~10^6 organisms per footpad by real-time in vivo imaging. From our experiments using an inoculum of 10^6 promastigotes, we have found that detection of infection by whole body imaging precedes that demonstrated by Vernier calipers by nearly 2 weeks. These advantages outweigh some of the inherent problems with the system, i.e. variability in the GFP fluorescence among individual parasites and diminishing plasmid copy number and fluorescence with the duration of time in vivo in the absence of selective pressure.

In conclusion, the application of GFP fluorescence for in vivo imaging provides a novel murine model of cutaneous leishmaniasis that allows for the evaluation of the
dynamics of ongoing infection in the same mice. In comparison with the classical footpad caliper measurements, we avoid the disadvantages of: 1) substantial variability associated with the current and routine caliper based methods that requires the use of large numbers of animals to obtain statistically reliable data; 2) measurement in a single vector to quantify a three dimensional infection; and 3) difficulty in differentiating extent of infection from local immune response. Our model is qualitative and semiquantitative, markedly more sensitive and precise than the standard caliper based method of following infection, and reduces experimental variation by allowing investigators to follow the same mouse through the course of an experiment, rather than sacrificing multiple groups of mice at serial time points. This powerful non-invasive whole-body imaging tool creates new opportunities for studying immunopathogenesis in murine leishmaniasis and for the evaluation of new prophylactic and therapeutic agents, and represents a significant refinement over previous animal models.

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Potential Conflicts of Interest: RMH is president of Anticancer, Inc. YG is an employee of Infectious Disease Research Institute. All other authors report no financial conflicts of interest.
Reference List


Figure 1: Photographs of a mouse footpad over time after inoculation with $10^7$ egfp transfected *L. amazonensis* promastigotes. Figures 1A-1F: On each pair of photographs, the left is taken using the fluorescence imaging system, while the right is taken using normal bright field imaging. The images are taken at seven day intervals as indicated in the figure with the Day 0 photograph taken just prior to inoculation.
(Photographs taken using OV-100® imaging system, Olympus, Japan). (5 mice were
used in this experiment, and one representative mouse was chosen for all of the
photographs). **Figure 1G:** A graph of the mean footpad measurement in mm (right axis)
with standard error over the course of the *Leishmania* infection (solid triangles). On the
same graph, the mean of the sum green pixel count (open circles) with standard error
from images taken at serial time points is plotted as well (left axis). GFP fluorescence,
indicating infection, was visualized when the first set of images was taken at day 7. A
significant difference in the mean measurements between day zero and each time point
was not reached until day 24 (p=0.01144)
Figure 2: Mouse footpads were inoculated with increasing numbers of egfp transfected Leishmania amazonensis from $10^2$ to $10^6$ promastigotes. Mice were followed over time, and footpad measurements and fluorescence imaging was done at regular intervals. Time of first detection of fluorescence for each inoculation was: $10^6$ – 4 days, $10^5$ – 18 days, $10^4$ – 32 days, $10^3$ – 42 days, and $10^2$ – 42 days. A. Graph of sum green pixel count in the fluorescence image at each time point for each inoculation dose. B. Graph of footpad
thickness at each time point for each inoculation dose. (Data from 2 mice averaged for each data point).
Figure 3: Figure 3a is a fluorescence micrograph of a tissue section taken 42 days after inoculation with a Nikon E600 at 1000X. The GFP-containing amastigote bodies are seen as the scattered green dots in the tissue section. Figure 3b a fluorescence micrograph taken at 400x of a touch prep of a mouse footpad infected for 5 weeks after inoculation with GFP transfected *L. amazonensis*. 3c is the same touch prep at the corresponding area of the slide taken at 400x stained with Hema 3®. Figure 3d is the 1000x magnification of inset 3d which demonstrates the presence of a *L. amazonensis* amastigote stained with Hema 3® that also corresponds to the fluorescent parasite in the bottom left corner of Figure 3b. Figure 3e is the 1000x magnification of inset 3e which...
demonstrates the presence of a *L. amazonensis* amastigote stained with Diff-Quick® that is again expressing GFP based on the fluorescence micrograph shown in 3b.
Figure 4: A. Images taken 2 hours after inoculation with increasing numbers of egfp transfected *L. amazonensis*. B. A graph of pixel counts of green fluorescence against the number of *Leishmania* inoculated, showing a linear relationship between infecting dose and pixel count (\( r = 0.9798 \) by Pearson method). Solid line represents best fit curve using linear regression.
A. Sum Green (pixels)

- Control
- Vaccine

p < 0.05 at Day 14

B. Footpad thickness (mm)

- Control PBS
- Leish 111f

p < 0.05 at Day 35

C. Images:

a

b
Figure 5: Immunotherapeutic suppression of *Leishmania* growth with Leish 111f + MPL-SE®. Fig. 5A is a graph of the sum green pixel count from mice inoculated with $10^7$ egfp transfected *L. amazonensis* promastigotes vs. time. Five mice in each group were imaged weekly. The mice in the control group received 25µl of PBS injected into the inoculated footpad at days 7, 14, and 24. The mice in the vaccine group received 100 µl of the Leish 111f + MPL-SE® vaccine divided between the right flank and the inoculated footpad at days 7, 14, and 24. Solid lines represent best fit curves using a non-linear regression exponential growth equation. Fig. 5B is a graph of the footpad measurements of the mice from the same experiment. The measurements of footpad thickness and GFP at each time point were compared between the control group and the treatment group and analyzed using the unpaired Student’s T-test. The difference in footpad measurements between groups did not reach statistical significance (p<0.05) until day 35, while the difference in GFP signal between groups was statistically significant at day 14. Fig. 5C shows representative photographs of a footpad from the control group –a and the vaccinated group- b at 24 days.