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Bioluminescent *Leishmania* expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harbouring macrophages and for quantitative real-time monitoring of parasitism features in living mice

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Summary

In this study, we have established conditions for generating *Leishmania amazonensis* recombinants stably expressing the firefly luciferase gene. These parasites produced significant bioluminescent signals for both *in vitro* studies and the development of an *in vivo* model, allowing the course of the parasitism to be readily monitored in real time in the living animals such as laboratory mice. First, a model was established, using parasite-infected mouse macrophages for rapidly determining the activity of drugs against intracellular amastigotes. Results indicated that recombinant *Leishmania* can be reliably and confidently used to monitor compounds acting on intracellular amastigote-harbouring macrophages. Secondly, temporal analyses were performed following inoculation of metacyclic promastigotes into the ear dermis of BALB/c mice and the bioluminescent light transmitted through the tissue was imaged externally using a charge coupled device (CCD) camera. Bioluminescent signals, measured at the inoculation site and in the draining lymph node of mice containing these parasites correlated well with the more classical quantification of parasites. These assays prove that the real-time bioluminescent assay is not only sensitive but also more rapid than culture-base techniques allowing to monitor parasite-load before any clinical signs of leishmaniasis are detectable. In short, this luciferase imaging study is useful to monitor the efficacy of anti-leishmanial drugs on live cell culture and to trace leishmanial infection in animal models.

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Introduction

Leishmania spp. are intracellular protozoan parasites transmitted from the sandfly during a blood meal to a variety of mammalian hosts. They cause a large spectrum of diseases affecting the skin mucous membranes and viscera. These dimorphic parasites exist as extracellular flagellated promastigotes in the insect vector and as obligatory intracellular amastigotes in the mammalian host. Experimental models, such as *in vitro* culture of mouse- or human-derived macrophages or laboratory mice, are largely used to study immuno-biology of these parasites and to screen the efficacy of newly developed drugs as well as vaccines (Blackwell, 1996; Launois *et al*., 1997; Handman, 2001). Simple, reliable and rapid test allowing the determination of drug activity at the mammalian stage are still needed for the development of new drugs and require detection and quantification of the *Leishmania* amastigote burdens in different host cells. This is routinely performed by Giemsa staining (Berman, 1984), culture-based techniques (Titus *et al*., 1985; Buffet *et al*., 1995), classic polymerase chain reaction (PCR)-based methods (Katakura *et al*., 1998; Noyes *et al*., 1998) or real-time quantitative PCR (Nicolas *et al*., 2002). However these techniques are cumbersome, laborious and rendered difficult the screening of several drugs at a time. Furthermore, while some of these methods are useful for *in vitro* studies, they have not proved ideal for the understanding of biological processes as they occur in living animals.

To better investigate parasite expansion, both *ex vivo* and *in vivo* in living cells, rapid, non-destructive, real-time monitoring methods that are adaptable to the experimental animal model are needed. To this end, reporter genes offer a method of labelling invasive microorganisms that is innocuous and allows the sensitive detection of only live, metabolically active cells by biophotonic imaging. The firefly luciferase represents one of the most efficient biological reporters. It catalyses the reaction of the substrate luciferin with adenosine triphosphate (ATP) to generate photons. Monitoring pathogenic processes in a living animal was first demonstrated with strains of Sal-

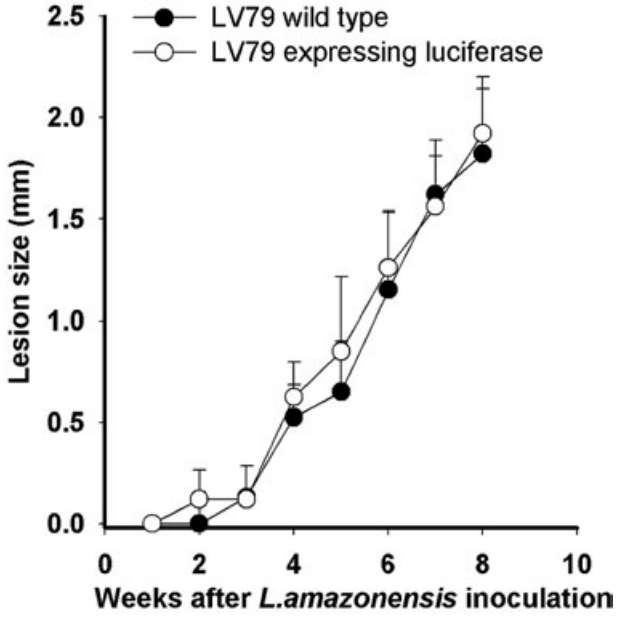

Fig. 1. Course of lesion development in BALB/c mice intradermally inoculated with 10^4 metacyclic promastigotes of *L. amazonensis*-wild-type strain or -expressing luciferase.

monella (Contag *et al.*, 1995). This non-invasive, rapid, real-time monitoring has been applied to various bacterial infection models and has been shown to have significant advantages other conventional methods for studying microorganism dissemination and treatment in animals (Loimaranta *et al.*, 1998; Francis *et al.*, 2000; 2001; Rocchetta *et al.*, 2001; Edinger *et al.*, 2003) or monitoring activities of bacteria within biofilm in a mouse model (Kadurugamuwa *et al.*, 2003). The use of luciferase gene has also improved the *in vitro* screening of antimicrobial agents acting on microorganisms developing within host cells (Deb *et al.*, 2000). The same strategy has been of great interest while testing new anti-leishmanial agents under both axenic and promastigote conditions (Luque-Ortega *et al.*, 2001; Sereno *et al.*, 2001).

In this study, we have established conditions for transfection of *Leishmania amazonensis* recombinants stably expressing the firefly luciferase gene. This luciferase expressing parasite was further analysed in two different systems: (i) *in vitro* drug screening on the clinically relevant stage of the parasites, i.e. amastigote-loaded mouse bone marrow derived macrophages, and (ii) the monitoring of the parasitic process in living animals. Thus, the light-emitting metacyclic promastigotes were injected into the ear dermis of BALB/c mice and the bioluminescent light transmitted through the tissue was imaged externally using a charge coupled device (CCD) camera at different time points after parasite delivery.

Results

Onset and development of lesions in BALB/c mice infected with bioluminescent L. amazonensis

Luciferase expressing-metacyclic promastigotes were injected into the ear dermis of BALB/c mice. The development of lesions was assessed by measuring the thickness of the ear over a period of 8 weeks (Fig. 1) and compared with the lesions induced by the inoculation of the same dose of wild-type promastigotes. In both cases, the onset of cutaneous clinical signs was detectable around week 3 and exhibiting ear lesions from week 7, which then steadily increased in size during the period examined (Fig. 1). This experiment clearly indicated that both control and luciferase-expressing parasites are driving the same clinical processes in BALB/c mice.

Bioluminescence assay on parasite populations at various developmental stages

To assess the feasibility of using bioluminescence as a quantitative indicator of the viability and the multiplication of parasites, studies were performed to compare bioluminescence to the number of viable parasites. Wild-type strain of *Leishmania* was used as control. After washing the cells, serial dilutions of parasites (2.5×10^6–1.2×10^3) were made to measure luciferase activity (Fig. 2).

The maximum level of bioluminescence was reached 5 min after substrate addition, followed by slow decay

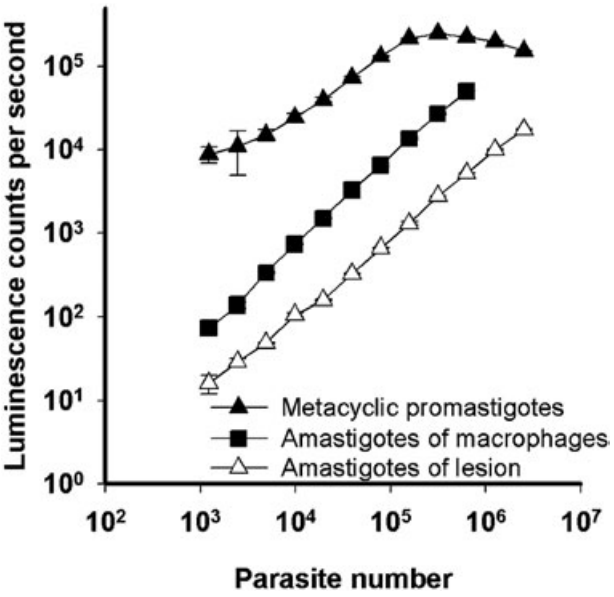

Fig. 2. Luminescence assay on luciferase-transfected *L. amazonensis*. Results for metacyclic promastigotes, amastigotes from BALB/c lesion or from infected macrophages are shown. The bioluminescent reaction was carried out as described in *Experimental procedures* with the Luciferase Reporter Gene Assay and the light emission was measured with the luminometer.

during 1 h. No bioluminescence (<10 cps) was observed in the wild-type strain at any the parasite stage (not shown), indicating that bioluminescence is specific to the luciferase-expressing parasites.

During log phase growth the luminescence signal was proportional to the number of bioluminescent parasites (data not shown). Once the cultures reached the stationary phase the bioluminescence decreased. This data suggests that a decrease in growth correlated with a decrease in metabolic activity of the population. When assayed with metacyclic promastigotes, luminescence (8.8×10^3 counts per second) was detected for 1.2×10^3 parasites, proportional to the cell number and reached 1.3×10^5 cps for 7.8×10^4 parasites (Fig. 2). We next investigated the bioluminescence of lesion-derived parasites and compared it with amastigotes purified from infected macrophages. Amastigotes were purified either from the *L. amazonensis*-infected footpads or from the *in vitro Leishmania*-harbouring macrophages. The luminescence obtained with lesion-derived amastigotes was 3–6 times lower than with amastigotes of macrophages and 10–50 times lower than with metacyclic promastigotes. The minimum level (47 cps for 1.2×10^3 amastigotes) was 10 times higher than the level measured with the wild-type strain. These results indicate that all the parasites recovered from lesions were luminescent confirming the stability of luciferase-transfected parasites in this type of lesion.

Validation of the luminescence assay using Leishmania*-infected macrophages: monitoring with drugs known to be active on* Leishmania *spp.*

Bioluminescence of *Leishmania* was measured in infected macrophages and inhibition was monitored in presence of anti-leishmanial drugs. Macrophages were infected with stationary-phase culture of transfected-*Leishmania* at an 1:8 multiplicity of infection in 24-well plate. The fate of *Leishmania* was then followed over a period of 48 h by measuring luminescence as well as the percentage of infected macrophages. The transfectants showed characteristics of the parental cells. They were infective for the macrophages and the differentiation into amastigotes with typical parasitophorous vacuole seen for *L. amazonensis* (Antoine *et al.*, 1991) was observed. The inhibition of growth by the anti-leishmanial compound amphotericin B was demonstrated. Over a period of 48 h, the percentage of infected macrophages at the time that the drug was added was high (>85%) and remained constant when no drug was added to the medium (Fig. 3A). However, after the treatment of macrophages with amphotericin, the percentage of infected cells and intensity of bioluminescence decreased proportionally to the drug concentration (Fig. 3A and B).

The high intensity and stability of bioluminescence of the luciferase-expressing *L. amazonensis* amastigotes

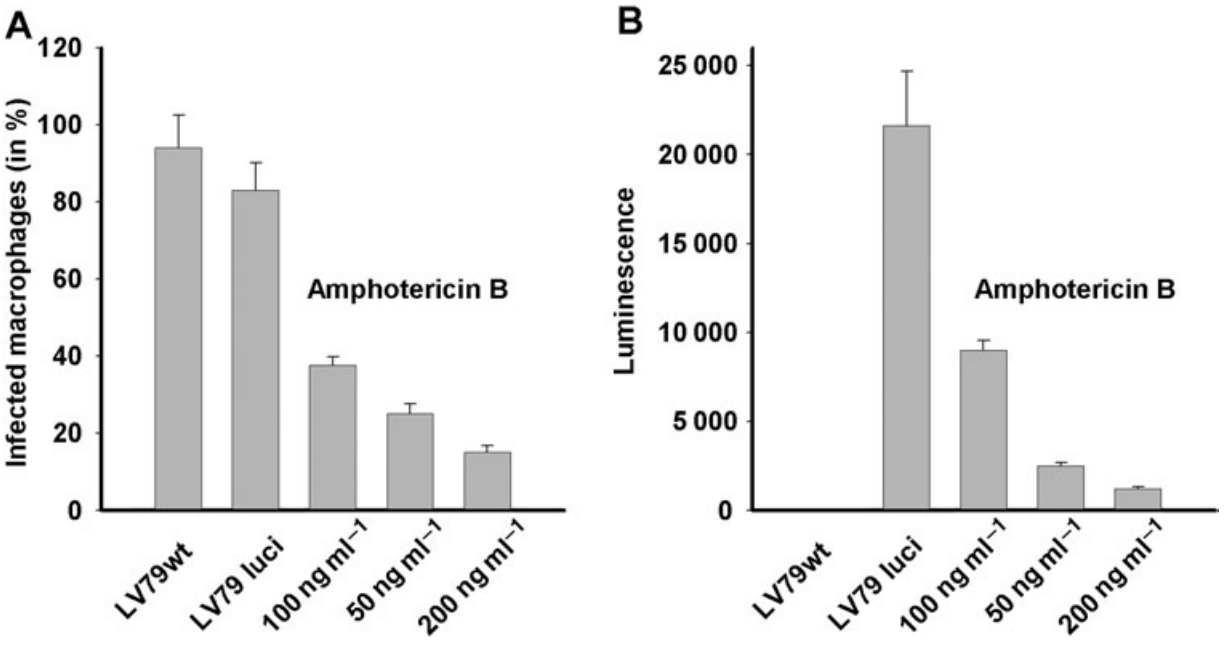

Fig. 3. Percentage (A) and bioluminescence intensity (B) of *Leishmania*-infected macrophages treated or not with amphotericin. Bone marrow-derived macrophages were prepared from BALB/c mice and infected with luciferase-transfected *L. amazonensis* promastigotes (eight parasites per host cell). Wild-type strain was used as negative control. Two hours later extracellular parasites were removed and after 24 h amphotericin B was added (200, 100, 50 ng ml^{-1}). The luciferase activity and the percentage of infected was measured 48 h after infection. The bioluminescent reaction in cells was triggered by the addition of the reagents for the determination of luciferase activity of the firefly, and the light emission was measured with a microtitration plate luminometer. The results are the mean of duplicates and representative of two experiments.

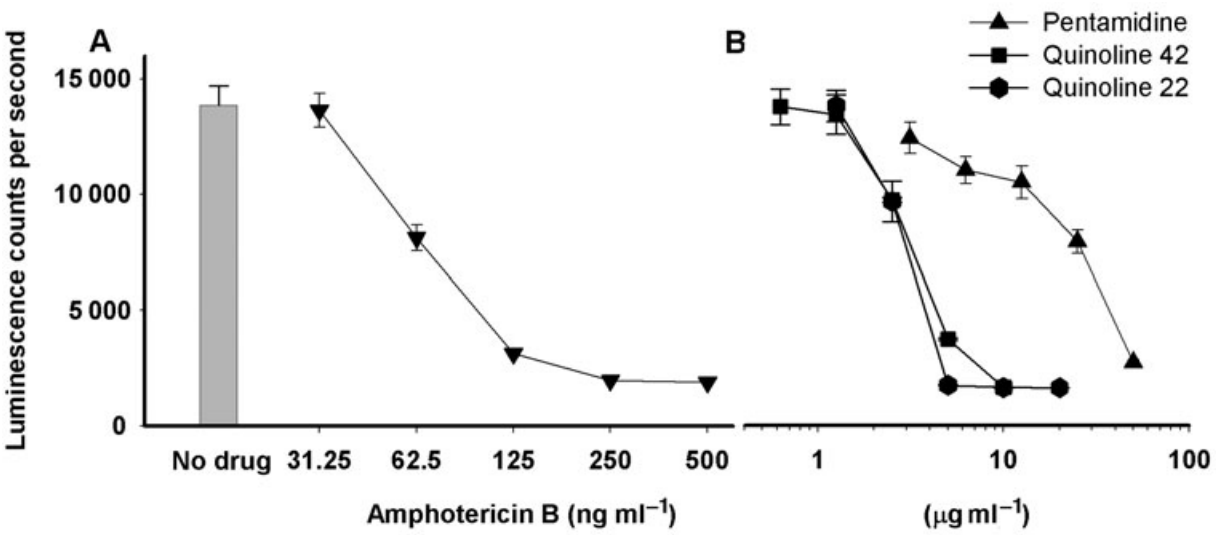

Fig. 4. Macrophages (4×10^4) were infected in 96-well plate with recombinant *Leishmania* and treated with amphotericin (A) or pentamidine (B) or quinolines (B). The drugs were added as described in Fig. 3 and the luciferase activity of infected cells was measured at 48 h with the Luciferase Reporter Gene Assay and the light emission was measured with the luminometer. The results are the mean of duplicates and representative of three experiments.

allowed drug testing in microplates. The inhibition of growth by the anti-leishmanial compounds amphotericin B, pentamidine or quinolines was demonstrated (Fig. 4). In both cases the difference in the level of luminescence corresponded to a difference in the percentage of infected cells, observed microscopically (not shown). These findings show that luciferase-expressing *Leishmania* could be useful in a bioluminescence assay for drug screening in 96-well plates.

Ex vivo *bioluminescence imaging of tissues from BALB/c mice inoculated with* L. amazonensis *in the footpad several months earlier*

After inoculation of 10^6 parasites in the footpad, small nodular lesions were detected in the mice from week 3; they increased quickly in size and displayed ulceration and tissue necrosis after 12 weeks. The onset of the cutaneous lesions was associated with a large increase in the number of parasites in the infected footpad ($>5 \times 10^8$ parasites). By contrast the number of parasites per draining lymph node remained low during the first weeks post-inoculation (about 100 parasites) and increased slowly in parallel with lesion progression until week 24 (10^4 parasites). No viable parasites could be recovered from the spleens during the onset of lesions and less than 500 parasites per spleen were detected by week 24 post-inoculation.

Mice primarily infected 24 weeks before in the footpad with 10^6 parasites received luciferin and were sacrificed. The tissues were surgically removed and bioluminescence signal was analysed at the site of inoculation and its draining lymph node as well as distant tissues including the spleen. Figure 5A illustrates the luminescent image of a representative lymph node; the bioluminescent signal was clearly detected and reached approximately 3×10^8 photons per second (Ph/s). By contrast the intensity of the bioluminescent signal in the footpad appeared to be lower (4×10^7 Ph/s; Fig. 5B) than that in the lymph node which could be due to the lack of both oxygen and luciferin to the wounded tissue. This last point was confirmed by the fact that the footpad exhibited a higher luminescence activity (3×10^8 Ph/s) when luciferin was added a second time in liquid medium (Fig. 5C). In the spleen the luminescence was not detectable.

In vivo *bioluminescence of* L. amazonensis *metacyclic promastigotes in living animals*

We next investigated the parasite burden during the first 2 weeks after parasite inoculation by determining the number of parasites present in infected ears and expressing them as limiting dilution assay units (LDAU) (Fig. 6). One day after the injection of 10^6 parasites, viable parasites were detected in the ear (about 2% of the inoculum). The number of parasites then increased slowly during the first week after inoculation and then increased substantially reaching 10^6 parasites by day 14. Successful infection, defined as the development of lesion in ear dermis, was observed in 100% of the inoculated mice.

Figures 7 and 8 illustrate the real-time luminescent images from representative mice, infected with luciferase-transfected parasites over a 16 day time-course. Mice were infected with 10^6, 10^5 or 10^4 parasites in the ear. Parasites produced a significant bioluminescent signal in

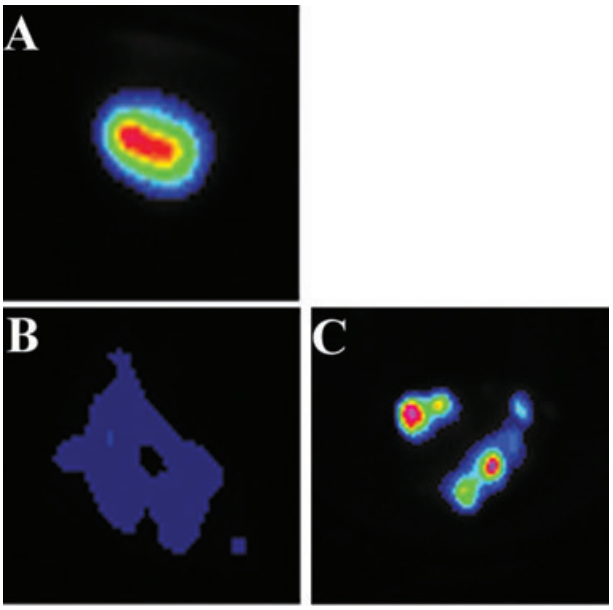

Fig. 5. *Ex vivo* imaging luminescence in the lymph node (A) and footpad (B, C) of BALB/c mice subcutaneously inoculated in the footpad with 1×10^6 luciferase-transfected *Leishmania amazonensis*. Six months after infection, luciferin was intraperitoneally injected in mice. Mice were then sacrificed and the lymph node and the footpad were removed before analysing by luminescence. In C, luciferin was added a second time directly in the medium. The bioluminescence was monitored by detecting photon emission using a CCD camera. The bioluminescent signal is presented as a pseudo-colour image representing light intensities (red most and blue least intense).

mice allowing the progression of infection to be monitored non-invasively in the experimental model. The total photon emission from the infected site was quantified and shown in Fig. 8. No bioluminescence was observed in uninfected

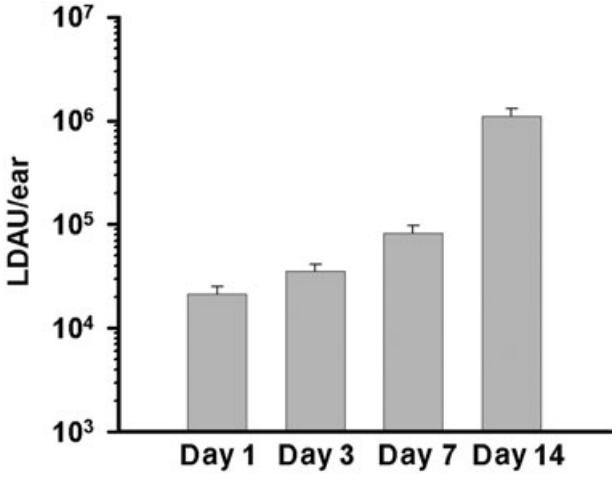

Fig. 6. Monitoring of parasite load in BALB/c mice inoculated intradermally in ear with 10^6 metacyclic promastigotes of *Leishmania amazonensis*. At various time-points after inoculation, parasite loads were estimated in the ears by limiting dilution assays. Results are expressed in LDAU per ear. Histograms show the mean values + one standard deviation for three individual ears.

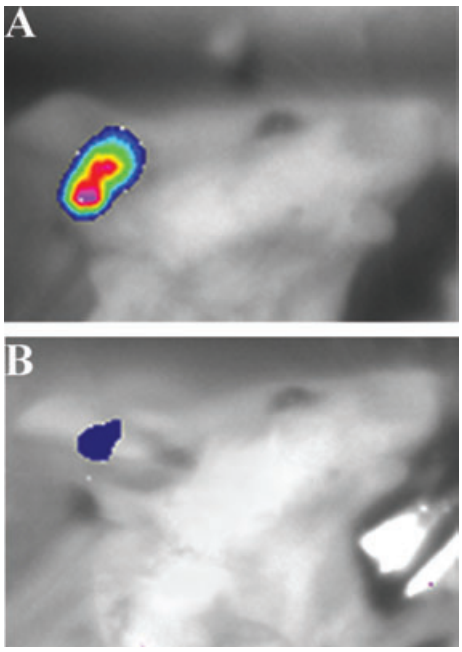

Fig. 7. *In vivo* luminescence imaging of *Leishmania* in a mouse model. Luciferase-transfected *Leishmania amazonensis* were inoculated at intradermal site with doses ranging from 1×10^6 (A) to 1×10^5 (B) and detection in the ear dermis was monitored by detecting photon emission at day 1 using a CCD camera. The bioluminescent signal is presented as a pseudo-colour image representing light intensities over the body surface area. Red represents the most intense light emission while blue correspond to the weakest signal. This pseudo-image is superimposed on the photography for orientation.

mice or mice infected with the wild-type strain. Following inoculation of transfected parasites, the bioluminescence measurements were determined at the inoculation site. Twenty hours after inoculation of 1×10^6 parasites, the bioluminescent signal was clearly detected at the inoculation site (Fig. 7A) and reached approximately 1.8×10^7 Ph/s (Fig. 8A). Successful infection, defined as the development of lesions in ear dermis, was observed in 100% of the inoculated mice. In the groups of mice that were inoculated with 1×10^5 *Leishmania* the infection rate was also 100% (not shown) but the bioluminescence was 15 times lower (1.2×10^6 Ph/s; Fig. 7B) than that in the group infected with the highest dose. In the last group of mice that were injected with 1×10^4 parasites the bioluminescence was not detectable and not significantly different of the background (4×10^5 Ph/s; not shown). Eight days after inoculation of 1×10^6 parasites, the bioluminescent signal remained stable (Fig. 8) and reached 2×10^8 Ph/s at day 16 after inoculation (Fig. 8A).

Discussion

Deciphering biological processes in the context of tissues, systems requires the development of non-invasive imaging modalities such as *in vivo* bioluminescence imaging

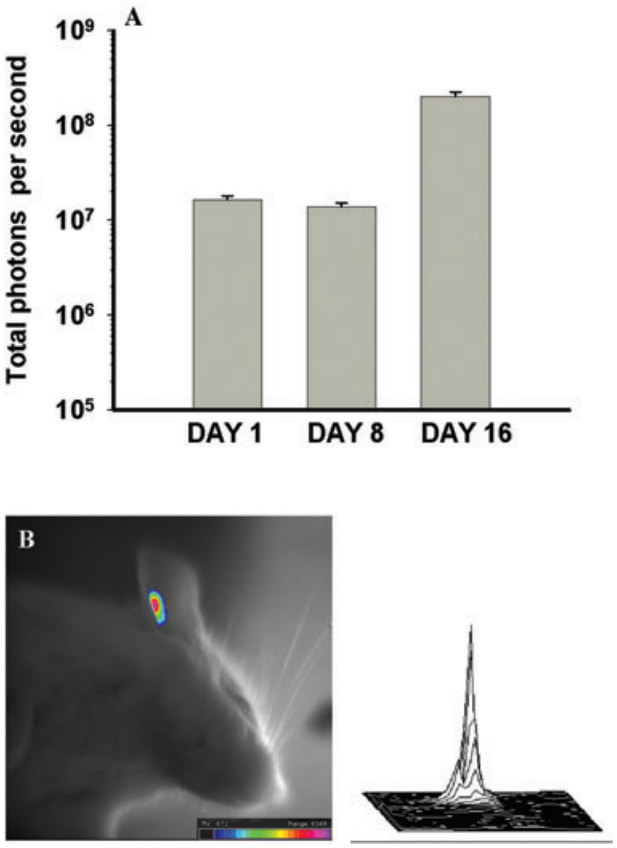

Fig. 8. Monitoring the level of bioluminescence activity on ear after intradermal inoculation of 10^6 luciferase-transfected *Leishmania amazonensis*. Images were acquired with CCD camera over a 16 day time course and (A) quantification of luminescence in the ear dermis at day 1, 8 and 16 was monitored by detecting total photon emission per second. The background obtained for the same area was subtracted to the measured values. (B) One image of infected mouse at day 8 is displayed as pseudo-colour image, with variation in colour representing light intensities as described in previous figure. Colour scale next to image codes for signal intensity. This image is superimposed on the reference image for localization. On right panel, the three dimensional distribution and relative amplitude of luminescence are shown at the inoculation point. The highest line, corresponding to the red area, represents the most intense light emission.

(Greer and Szalay, 2002; Doyle *et al*., 2004). In the present study we aimed to detect and track bioluminescent parasitic microorganisms through the course of the parasite/host interactions using the firefly luciferase as the reporter gene as well as its substrate the luciferin. The stable integration of the luciferase reporter gene into the ribosomal locus of *L. amazonensis* represents a valuable tool for (i) parasite targeted-drug discovery and testing, and (ii) assessing the discrete phases occurring in the different hosts they rely on.

Setting-up of standard curves for each environmental condition: the example of parasites displaying different developmental stages

Leishmania spp. display different developmental stages that assess their reactivity to the host signals they rely on: while they develop as extracellular flagellated promastigotes in the insect vector, they develop as an obligate intracellular amastigotes that survive and multiply within the mammalian host. The stable integration of the luciferase reporter gene into the ribosomal locus of *L. amazonensis* represents a valuable tool for assessing phenotypes related to parasite features in laboratory mice when long periods of growth without selection is required. The measure of luciferase activity was compared and the level of sensitivity determined using recombinant parasites at different stages of differentiation and purified from culture or mouse lesions. It was possible to detect 10 times more luciferase-expressing amastigotes parasites extracted from cultured bone marrow-derived mouse macrophages, compared to amastigotes purified from mouse cutaneous lesions. Similarly when luciferase-expressing amastigotes derived from cultured macrophages, are differentiating into promastigotes, for the same number of parasites, we observed a 30-fold increase in luciferase activity in the latter. Several factors can contribute to lower luciferase activity in amastigotes and intracellular parasites such as their slower metabolism, different environmental conditions such as medium, protease inhibitors. Whatever the reasons are, the correlation between luciferase activity and parasite number was excellent both in culture and in infected macrophages or mouse tissues: as a result, standard curves for each condition have been set up and the level of sensitivity is relevant for most purposes and reproducible within one defined condition. The minimum number of parasites (amastigote stage) detected by their bioluminescent phenotypes was 1000 and in this study we show that the linear relationship of bioluminescence versus number of parasites was shown to give a dynamic measurement range of over four logs. Furthermore, it is possible to use luciferase activity derived from a standard curve established with purified amastigotes to determine parasite burdens in macrophages or in mouse tissues.

Setting-up of relevant readout assays for parasite targeted-drug discovery and testing

For a number of intracellular pathogenic microorganisms, the use of reporter genes has facilitated antimicrobial drug discovery and testing (Jacobs *et al*., 1993; Buckner *et al*., 1996; Collins *et al*., 1998; Deb *et al*., 2000; Chan *et al*., 2003). Recently, models of axenic *Leishmania* amastigotes have been introduced and proposed for drug testing, instead of promastigotes (Callahan *et al*., 1997; Ephros *et al*., 1999; Sereno *et al*., 2001). Still these axenic amastigotes differ from the amastigotes that either develop in unactivated/de-activated macrophages (Callahan *et al*., 1997; Sereno *et al*., 1998; Sereno *et al*., 2001) or that persist in asymptomatic cutaneous tissue.

In this study the activities of anti-leishmanial drugs against *Leishmania* sequestered in mouse bone marrow-derived macrophages were evaluated by direct assay of the inhibition of luminescence. In order to validate the system this latter measure was compared with their effect on the evaluation of amastigote number in macrophage. The major difference between these two methods is that luciferase method measures the total number of parasites present while the staining method is based on the regression of parasitophorous vacuole (Fakhfakh *et al*., 2003) and the overall decrease in parasite number. Inhibition of luminescence correlated well with the leishmanicidal activity of various drugs tested. This level of this inhibition at the same concentration of drug was always similar to the inhibition of parasitophorous vacuole. In our conditions the level of sensitivity was sufficient to detect the presence of home parasite per 10 macrophages in a 96-well plate containing 4×10^4 host cells. Thus, luciferase-expressing parasites offer many advantages over alternative drug screening methods. This experimental system has merits, as it is relatively fast and inexpensive, and has potential for automation and high throughput drug screening. It will allow the rapid identification of candidate compounds that are suitable for further testing on amastigotes. In conclusion, from the data reported in this paper, it seems likely that recombinant *Leishmania* expressing reporter gene might be reliably and confidently used for the screening of new compounds active at least on DNA-replicating amastigotes that develop in mouse macrophages.

Imaging of bioluminescent parasites in mice: one way to visualize features of parasitism?

The next challenge was to dispose a direct assessment of the features of parasitism in real time. Does this technique provide both spatial and sequential information about the features of parasitism within the tissue context? Because of its non-destructive and non-invasive nature the imaging procedure can be performed repeatedly, allowing each animal to be used as its own control over time, overcoming the problem of animal–animal variations. Moreover, these *in vivo* methods do not require the removal of tissue and consequently the loss of contextual influences of the living animals (Contag *et al*., 1995; Francis *et al*., 2000; 2001; Rocchetta *et al*., 2001; Luker *et al*., 2002). More and more studies (Contag *et al*., 1995; Francis *et al*., 2000; 2001; Rocchetta *et al*., 2001; Luker *et al*., 2002) indicate not only that *in vivo* bioluminescent imaging is feasible but also that it can be quantitative and providing more information in less time than conventional assays. However, as far as the eukaryotic parasites are concerned, detection devices to visualize and quantify optical signal in living cells are still limited to *in vitro* or *ex vivo* analyses. Thus, *Leishmania*, transfected with reporter genes, has been used in cell culture (Misslitz *et al*., 2000; Roy *et al*., 2000) and in *ex vivo* analyses after expression in animals (Misslitz *et al*., 2000; Roy *et al*., 2000; Chan *et al*., 2003; Sorensen *et al*., 2003). The present study is the first demonstration of detection and monitoring light emission in a murine model of parasitical infection. First, it was established that (i) the features of the parasites that have been transfected are not different from the non-luciferase expressing ones on the basis of the onset and the development of the lesion, and (ii) the intensity of the bioluminescence measured from the living mice correlated well with the parasite burden subsequently determined by standard protocols. Second, in the present study we observe that photon count imaging and the estimate of the cultivatable parasites increase in the same manner. Consequently, the bioluminescence could be used to monitor the parasitic load at the site of inoculation. In addition, following the intradermal delivery of low doses of invasive metacyclic promastigotes (Courret *et al*., 2003), the onset of the lesion in BALB/c mice was shown to assess the presence of doses as low as 10^4 parasites. The non-invasive approach we describe in the present paper could be further coupled to the use of mouse hosts expressing relevant bioluminescent probes especially if the sensitivity of the equipment is improved (Doyle *et al*., 2004).

In conclusion, this study presents a new model for visualizing the different discrete phases that *Leishmania* establishes in one of its hosts, the laboratory mice. The method described here is rapid, uses fewer animals and enables *in vivo* monitoring of the parasite burden. Additionally, this model may be useful for the study of pathogenesis and healing processes in mice, allowing to dissect one essential feature of parasitism, namely the parasite persistence, and its transmissible properties to the insect vector.

Experimental procedures

Mice

We obtained 2 to 4 month old female BALB/c from Charles River (St Germain-sur-l'Arbresle, France).All the animal were handled under institutional guidelines of the Central Animal Facility at Institut Pasteur (Paris, France; http://www.pasteur.fr/recherche/unites/animalerie/guidelin.html).

Generation of bioluminescent Leishmania

A 1.66 kbp of *firefly luciferase* coding region was cut by NcoI/EagI from pGL3 basic (Promega, Madison, WI) and subsequently cloned into *Leishmania* expression vector pF4× 1.HYG (Jenabioscience, Jena, Germany) with marker gene for selection with Hygromycin B (Cayla, Toulouse, France) and previously cut by NcoI/NotI. In this construct 18 s rRNA flanked the luciferase and HYG genes. Following linearization with SwaI, luciferase and

HYG was integrated into 18 s rRNA locus of the nuclear DNA of *Leishmania.*

Transfection were realized by electroporation with following conditions: 25 μF, 1500 V, in 4 mm cuvette; 3.75 kV cm^{-1} (Goyard *et al.*, 2003). Following electroporation, cells were incubated 24 h in media without drug, and plated on semisolid media containing 100 μg ml^{-1} of hygromycin B.

Parasite preparation and inoculation in mice

Wild-type or transfected-*L. amazonensis* strain LV79 (MPRO/BR/1972/M1841) amastigotes were prepared from BALB/c mice (Antoine *et al.*, 1989). Briefly, the promastigote form was grown at 26°C in M199 media supplemented with 10% FBS, 25 mM Hepes pH 6.9, 12 mM $NaHCO_3$, 1 mM glutamine, 1× RPMI 1640 vitamin mix, 10 μM folic acid, 100 μM adenosine, 7.6 mM haemin, 50 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin (Goyard *et al.*, 2003). Infective-stage metacyclic promastigotes were isolated from stationary phase cultures (6 days old) by gradient of Ficoll as previously described (Spath and Beverley, 2001). Various doses (10^4–10^6) of metacyclic promastigotes per 10 μl of Dulbecco's PBS were injected in the ear dermis of BALB/c mice. Lesion size was measured using a direct reading Vernier caliper (Thomas) and expressed as the difference in thickness between the inoculated ear and the non-inoculated contra-lateral ear.

Quantification of parasites

We estimated the number of parasites present in parasite-loaded ears and the draining lymph nodes, as previously described (Leclercq *et al.*, 1996). The two sheets of the infected ears were separated, cut into small pieces and ground in HOSMEM-II culture medium, using a glass tissue homogenizer. Lymph nodes were removed, mechanically dissociated and the number of cells they contained was determined. The tissue/organ homogenates were serially diluted in HOSMEM-II culture medium and then dispensed into 96-well plates containing semisolid agar (Bacto-Agar, Difco, Detroit, MI) supplemented with 10% sterile heparin-treated rabbit blood. The plates were incubated for 10 days and then each well was examined and classified as positive or negative according to whether or not viable promastigotes were present. Limiting dilution analysis was then applied to the data to estimate the number of viable parasites, expressed in LDAU. Statistical analysis of the results was based on the maximal likelihood method (Taswell, 1981; Strijbosch *et al.*, 1987).

Macrophage infections and parasites drug susceptibility assay

Macrophages were obtained from BALB/c mice by *in vitro* differentiation of bone marrow precursor cells in bacteriologic Petri dishes (Greiner, Nurtingen, FRG). Cells were cultured in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 20% (v/v) heat-inactivated fetal calf serum (Dutscher, Brumath, France), 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 20% L-929 fibroblast-conditioned medium. After 5 days at 37°C in a 5% CO_2/95% air atmosphere, cells were washed in PBS and removed by treatment for 20 min with Ca^{2+}, Mg^{2+}-free PBS containing 2 mg ml^{-1} glucose (Prina *et al.*, 1993). Recovered macrophages were further incubated in culture medium containing only 3% of conditioned medium either in 24- or 96-well plates or 35 mm culture dishes. Twenty-four hours after re-plating, macrophages were infected at a stationary phase promastigote–host cell ratio of 8:1 and then incubated at 34°C for 2 h before being washed with Dulbecco's phosphate-buffered saline (PBS, Seromed) to remove free parasites. Following 1 day incubation to allow differentiation in amastigotes, drug was added to the appropriate wells diluted in RPMI with 20% FBS and cells were incubated at 34°C for a further incubation of 24 h. For quinolines, compounds 22, 42 were used and the stock solutions were prepared in DMSO as described previously (Fakhfakh *et al.*, 2003). Macrophages were either fixed and stained with Giemsa to assess the level of infection by light microscopy or directly treated for the quantification of bioluminescence. The percentage of amastigotes-loaded macrophages and the number of parasites per macrophages were determined by looking at least 200 macrophages in two independent wells.

Extraction of intracellular parasites from macrophages and their further processing

Macrophages on 35 mm tissue cultures dishes were washed with PBS and then lysed in CHAPS lysis buffer (20 mM Tris-HCl buffer, pH 7.4, containing 130 mM NaCl and 0.3% (w/v) CHAPS (3[(3-chloroamidopropyl)dimethylammonio]-1-propane sulphonate, Sigma) (Courret *et al.*, 2001).

After 40 min at 4°C, macrophages were scraped off the dish using a rubber policeman. Recovered cells were collected and intracellular parasites were purified through a 45%/90% Percoll gradient (Pharmacia, Uppsala, Sweden). Viable parasites were washed and counted and prepared for luciferase assay.

*Estimation of luciferase content in transfected-*Leishmania *promastigotes and in infected macrophages*

Parasites were washed in PBS and transferred in 96-well white culture plate (Dynex Technologies, Chantilly, France) to prevent cross-talk between wells during detection. Various doses of parasites were incubated with the luciferase assay system kit (Roche Molecular Biochemicals; Mannhein, Germany) for 5 min at room temperature. During this time complete cells lysis occurred and the light reaction started. Then plates were transferred to the Wallac MicroBeta Trilux scintillation counter (PerkinElmer Life Sciences, Boston, MA) used as luminometer and the light reaction was measured for 1 s. The luminescence units are luminescence counts per second (LCPS). In measuring luminescence the lower of the photomultiplier tubes (PMTs) is turned off and measurement is accomplished by the upper PMT only. Untranfected parasites were used as negative control. *Leishmania*-infected macrophages were washed with PBS. For measurement of bioluminescence, 100 μl of PBS was added to each well of macrophages and mixed with 100 μl of luciferase reagent.

In vivo *bioluminescence imaging of luciferase-expressing* Leishmania

The mice were anesthetized by intraperitoneal administration of

ketamine (120 mg kg^{-1}; Imalgène 1000, Mérial, France) and xylazine (4 mg kg^{-1}; Rompun 2%, Bayer, Leverkusen, Germany). A solution of the luciferase substrate luciferin (150 mg kg^{-1}; D-Luciferin potassium salt, Xenogen, California) was mixed with the anaesthetics just before use. A greyscale reference image of animals was generated and mice were imaged for a maximum of 5 min at a number of time points post-infection using a NightOwl cabinet with charge couple device (CCD) camera (Berthold Technologies, Bad Wildbad, Germany). Total photon emission from selected and defined areas within the images of each mouse was quantified with the WinLight32 software (Berthold). The photon signal from the ear was presented as a pseudocolour image representing light intensity (red most and blue least intense). This image was superimposed on the reference image for orientation.

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