Parasitology International xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

In vivo imaging of trypanosomes for a better assessment of host–parasite relationships and drug efficacy ☆

S. Goyard, P. Lourenço Dutra [1], P. Deolindo [2], D. Autheman, S. D'Archivio, P. Minoprio *

Institut Pasteur, Laboratoire des Processus Infectieux à Trypanosomatidés, Département Infection et Epidemiologie, 25 rue du Dr. Roux, 75724 Paris, France

ARTICLE INFO

Article history:
Received 31 October 2012
Received in revised form 20 May 2013
Accepted 16 July 2013
Available online xxxx

Keywords:
Trypanosoma cruzi
Trypanosoma vivax
In vivo imaging
E2-Crimson
GFP
Firefly luciferase
Bioluminescent imaging

ABSTRACT

The advances in microscopy combined to the invaluable progress carried by the utilization of molecular, immunological or immunochemical markers and the implementation of more powerful imaging technologies have yielded great improvements to the knowledge of the interaction between microorganisms and their hosts, notably a better understanding of the establishment of infectious processes. Still today, the intricacies of the dialog between parasites, cells and tissues remain limited. Some improvements have been attained with the stable integration and expression of the green fluorescence protein or firefly luciferase and other reporter genes, which have allowed to better approach the monitoring of gene expression and protein localization *in vivo*, *in situ* and in real time. Aiming at better exploring the well-established models of murine infections with the characterized strains of *Trypanosoma cruzi* and *Trypanosoma vivax*, we revisited in the present report the state of the art about the tools for the imaging of Trypanosomatids *in vitro* and *in vivo* and show the latest transgenic parasites that we have engineered in our laboratory using conventional transfection methods. The targeting of trypanosomes presented in this study is a promising tool for approaching the biology of parasite interactions with host cells, the progression of the diseases they trigger and the screening of new drugs *in vivo* or *in vitro*.

1. Introduction

Neglected diseases have gained particular attention from the economic powers these last years. These diseases are caused by parasites, bacteria or viruses considered until recently as those whose research support is very limited in comparison with the immense burden the diseases they cause, pose to humanity [1]. Today, one-sixth of the world's population suffers from at least one of these pathologies, as stated by the Global Health Observatory from the World Health Organization. They are not a "market" attractive to stimulate the large pharmaceutical companies in research and development (R&D) of new therapies. Not long ago, some efforts have revived the interest of pharmaceutical companies for "neglected" diseases, mainly targeting malaria, tuberculosis and AIDS (Acquired Immunodeficiency Syndrome). Since these three diseases are present in rich countries or affect travelers, these efforts are supported by financial incentives that include mixed partnerships between the public and private sectors. Unfortunately, this strategy has little impact on what is now described as the "most neglected diseases" such as those caused by parasites from the *Trypanosomatidae* family comprising Human and Animal trypanosomosis, leishmaniosis and Chagas Disease. The socio-economic impact of these diseases is very considerable, even if it tends to decrease in response to advances in the fight against insect vectors. The increased knowledge of cell biology and of the metabolic pathways of these trypanosomatids, as well as a more detailed knowledge of the interaction of these parasites with their hosts may carry new rational approaches to improve existing chemotherapies. In addition, the discovery of new chemotherapeutic targets against these microorganisms is a major goal of the Millennium that relies on the setting up of better and reliable medium and high throughput drug screening assays.

Some improvements to these goals have been anticipated in this last decade from the techniques of transfection developed for parasites using several reporter genes. For instance, they have highly contributed for the understanding of many crucial steps of the biology of the microorganisms and their relationship with their hosts at cellular and tissue levels. Pioneer studies on the expression of foreign intracellular reporter genes by trypanosomatids date back to the 90s. Early successful attempts started in 1991 with the transient expression of β-galactosidase (*β-gal*) and β-glucuronidase as reporter enzymes in *Leishmania (L.) tropica* [2]. The consideration of glycosome biogenesis to approach the energy source in trypanosomes was first studied in 1992 by the stable expression of firefly

* Corresponding author. Tel.: +33 1 4568 8615; fax: +33 1 4061 3185.
E-mail address: paola.minoprio@pasteur.fr (P. Minoprio).
[1] Present address: Departamento de Microbiologia, Imunologia e Parasitologia Universidade do Estado do Rio de Janeiro- UERJ, Rua Prof. Manuel de Abreu, 444, Rio de Janeiro, RJ 20550-170, Brasil.
[2] Present address: Laboratório de Biologia Molecular de Parasitas e Vetores, Instituto Oswaldo Cruz-FIOCRUZ, Avenida Brasil, 4365 - Manguinhos, Rio de Janeiro, RJ 21040-900, Brasil.

1383-5769/$ – see front matter © 2013 The Authors. Published by Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.parint.2013.07.011

luciferase by procyclic forms of *Trypanosoma (T.) brucei* [3]. These studies, were followed by others using episomal or stable expression of other enzymes or green fluorescent protein (GFP) genes, to tackle gene expression and protein targeting in *Leishmania major* and *Trypanosoma cruzi* [4,5]. These reporter genes have been progressively used since then because they are sensitive, inexpensive, present low toxicity, and they are not expressed by mammalian cells. One of the best advantages of the utilization of transformed parasites with these genes is that they do not require any fixation and most of them allow an easy imaging *in vitro* and *in vivo*. For instance, different strains of *T. cruzi* were later transfected with an integrative vector that allows the expression of GFP or red fluorescent protein (RFP) showing the usefulness of these mutants to study several aspects of the infection such as the mechanisms of cell invasion, of genetic exchange and the differential tissue distribution in animal models [6]. These studies were followed by those using trypomastigote and amastigote forms of *T. cruzi* expressing the luciferase (*luc*) reporter gene illustrating the advantages of bioluminescent parasites for the imaging *in vitro* and *in vivo* [7]. The disease progression of experimental Human African Trypanosomosis and the systemic tropism, particular to the brain, were subsequently demonstrated by bioluminescent imaging using mouse adapted strains of *Trypanosoma brucei gambiense* [8]. Our recent studies with metacyclic trypomastigote forms of *Trypanosoma vivax* expressing luciferase have reinforced the presence of the parasite in the central nervous system of mice and consequently the brain commitment in the very late phases of the experimental infection [9]. Other colleagues, using intravital brain imaging and *Trypanosoma brucei brucei* or *Trypanosoma brucei rhodesiense* parasites, respectively expressing mOrange and tdTomato, have equally reported that human trypanosomes can invade the murine brain parenchyma during the early stages of infection before meningoencephalitis is fully established [10]. Interestingly, *T. b. brucei* expressing luciferase has allowed the determination of the commitment and dissemination of the parasites to the testis observation that may hamper the efficacy of treatments for sleeping sickness [11].

Other advances have been reported about the utilization of reporter genes in protozoan parasites for the discovery and development of new drugs and of the *in vivo* imaging for noninvasive visualization of biological processes (see [12] for a review). Some examples include the efficacy of drug screening based on the use of GFP-expressing *L. major* or *Leishmania donovani* [13,14], EGFP-, *β-gal*- or *luc*-expressing *Leishmania amazonensis* [15,16] and more recently on *T. cruzi* expressing the tomato protein [17]. In the present work we report some of the new tools we have developed in our laboratory to the well characterized strains of *T. cruzi* (CL and Y) and *T. vivax* (ILRAD 1392), aiming at better exploring their infectious process at cellular and tissue levels and to evaluate the effects of new therapeutic compounds *in vitro* and *in vivo*.

2. Material and methods

2.1. Mice and ethics

Seven to ten week-old male Swiss Outbred mice (CD-1, RJOrl: SWISS) (Janvier, France) were used in all experiments. All mice were housed in our animal care facility in compliance with European animal welfare regulations. Institut Pasteur is a member of Committee #1 of the Comité Régional d'Ethique pour l'Expérimentation Animale (CREEA), Ile de France. Animal housing conditions and the procedures used in the work described herein were approved by the "Direction des Transports et de la Protection du Public, Sous-Direction de la Protection Sanitaire et de l'Environnement, Police Sanitaire des Animaux" under number B 75-15-28, in accordance with the Ethics Charter of animal experimentation that includes appropriate procedures to minimize pain and animal suffering. PM is authorized to perform experiments on vertebrate animals (license #75-846 issued by the Paris Department of Veterinary Services, DDSV) and is responsible for all the experiments conducted personally or under her supervision as governed by the laws and regulations relating to the protection of animals. All animal work was conducted in accordance with relevant national and international guidelines.

2.2. Parasite strains and cell cultures

T. (Dutonella) vivax IL 1392 was originally derived from the Zaria Y486 Nigerian isolate [18]. These parasites have recently been characterized and are maintained in the laboratory by continuous passages in mice, as previously described in detail [19,20]. For *in vivo* experiments, mice were injected intra-peritoneally or sub-cutaneously with bloodstream forms of *T. vivax* (10^2 parasites/mouse). Parasitemia was determined, as previously described [19]. Cell culture-derived or axenic amastigote and trypomastigote forms from *T. cruzi* CL Brener (clone F11–F5) (www.dbbm.fiocruz.br/TcruziDB/clbrener.html) and Y strains were isolated from the supernatant of bulk cultures of green monkey Vero kidney cells previously infected with bloodstream trypomastigotes [21]. Epimastigote forms of *T. cruzi* are maintained by serial passages *in vitro*, as previously described [22]. Normal or infected Vero cell cultures were seeded in microplates or in flasks at 5×10^4 cells/ml in RPMI 1640 medium/5% FCS and kept at 37 °C, 5% CO_2.

T. vivax metacyclics were purified from epimastigote axenic culture according to [23] with minor modifications. Normal goat serum was added to culture supernatants to a final concentration of 10% and incubated for 30 min at 27 °C. During the incubation period, epimastigotes aggregated and formed clumps, while metacyclics remained swimming freely. The metacyclics were then separated from the epimastigote clumps by differential centrifugation using a swing out rotor (Jouan GR412, Fisher Bioblock Scientific, Strasbourg, France) for 5 min 200 g.

2.3. Vectors and transgenic parasites

The nourseothricin resistance gene (SAT) was amplified from pFX4.1SAT (Jena Bioscience) with the primers SAT-forward (5′ ATGGC GCGCCATGAAGATTTCGGTGATCC 3′) and SAT-reverse (5′ CGCCATGGTT AGGCGTCATCCTGTGCTCC 3′). The fragment obtained (539 bp) was digested with AscI and NcoI and inserted into AscI and NcoI sites of the p*Tv*LrDNA-luc vector [20] to replace the neomycin resistance gene to obtain p*Tv*LrDNA-luc-SAT (see Table 1). The gene encoding the near infrared fluorescent protein E2-Crimson [24], was amplified

Table 1
Plasmid characteristics.

Plasmid	Species	Size (bp)	Promoter	5′UTR trans-splicing region	Reporter gene	Intergenic region	Selectable marker	3′ UTR
pTvLrDNA-luc [20]	*T. vivax*	8390	18S r DNA[a]	*Tv*PRAC	Luciferase	*αβ-tub*	*Sat*	*βα-tub*
pTvLrDNA-crim	*T. vivax*	7197	18S r DNA[a]	*Tv*PRAC	Crimson	*αβ-tub*	*Sat*	*βα-tub*
pTcTREX-crim	*T. cruzi*	6929	18S r DNA[b]	*HX1-TcP2β*	Crimson	*5′-gapdh*	*Neo*	*3′-gapdh*
pTcTREX-luc	*T. cruzi*	8164	18S r DNA[b]	*HX1-TcP2β*	Luciferase	*5′-gapdh*	*Neo*	*3′-gapdh*
pTcTREX-gfp [25]	*T. cruzi*	7027	18S r DNA[b]	*HX1-TcP2β*	GFP	*5′-gapdh*	*Neo*	*3′-gapdh*

[a] 1.8 kb fragment upstream the 18S rDNA of *T. vivax*.
[b] 0.8 kb fragment upstream the 18S rDNA of *T. cruzi*.

Please cite this article as: Goyard S, et al, *In vivo* imaging of trypanosomes for a better assessment of host–parasite relationships and drug efficacy, Parasitol Int (2013), http://dx.doi.org/10.1016/j.parint.2013.07.011