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Research brief

Trypanosoma brucei 29–13 strain is inducible in but not permissive for the tsetse fly vector

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Abstract

Using green fluorescent protein as a reporter, we have shown that the strain 29–13 of *Trypanosoma brucei*, widely used for inducible down-regulation of mRNA, is inducible in, but not permissive for the tsetse flies *Glossina palpalis gambiensis* and *Glossina morsitans morsitans*. Within two weeks post-infection, 42% males and females of teneral and non-teneral tsetse flies harboured intestinal infections, yet not a single infection progressed into the salivary glands.

Index Descriptors and Abbreviations: *Trypanosoma brucei*; Tsetse; *Glossina*; GFP; Transmission; Midgut infection; Tetracycline induction

Trypanosoma brucei, the causative agent of African sleeping sickness in humans and nagana in animals, became one of the first organisms in which RNA interference (RNAi) was shown to be effective (Ngo et al., 1998). Moreover, thanks to a tight and efficient inducible system (Wirtz et al., 1999), the target transcripts can be down-regulated by the addition of tetracycline (Tet) to the growth medium. Unexpectedly, *Trypanosoma cruzi* and *Leishmania major*, the other two medically important kinetoplastid flagellates for which the whole genome sequence is available (El-Sayed et al., 2005), are so far not amenable for the techniques of RNAi (Motyka and Englund, 2004). Therefore, *T. brucei* became the favourite flagellate for studies of gene function, with hundreds of genes already studied by means of RNAi (Subramaniam et al., 2006).

The synthesis of double stranded RNA can be efficiently induced in both procyclic and bloodstream stages of *T. brucei* under cultivation conditions and most functional studies have thus been performed in the culture media. Such conditions enable biochemical and molecular studies of gene functions, but at the same time are artificial and can hardly replace experiments with these parasitic stages in their respective hosts. It was shown that in the blood of mice fed with doxycycline, an analogue of Tet, RNAi can be triggered enabling the study of ensuing phenotypic effects (Krieger et al., 2000). Moreover, Tet-inducible gene expression has been recently demonstrated also within the tsetse fly vector. The expression of green fluorescent protein (GFP) was successfully induced in the *T. brucei* strain K11 by feeding 1 μg/ml Tet to tsetse flies *Glossina morsitans* 12 days after they have been infected with the bloodstream forms (Peacock et al., 2005). Also, the transgenic *T. brucei* strain AnTat 1.1 with genes knocked out by homologous recombination has been tested for possible

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phenotypes in the tsetse flies (Vassella et al., 2000; Urwyler et al., 2005, 2007). Lately, genetically modified cell lines derived from the strain Lister 427 have been assayed for phenotypes confined to the midgut infection (Nagamune et al., 2004; Guther et al., 2006), as some data indicated that this strain may not be able to produce mature infection in *G. morsitans* (Liniger et al., 2004).

Most functional analyses are being performed via RNAi, all making use of the *T. brucei* strain 29–13 strain. This is because only this strain, derived from the strain Lister 427 (Wirtz et al., 1999), contains both Tet-inducible system and viral T7 RNA polymerase and can be efficiently transformed with a number of different RNAi vectors (Motyka and Englund, 2004). We therefore undertook a study with the aim of testing the inducibility and permissibility of the 29–13 strain for *Glossina* spp.

Our preliminary experiments confirmed previous observations (Peacock et al., 2005) that feeding the tsetse flies with blood containing 1 μg/ml Tet does not compromise their viability and longevity (data not shown). The drug-resistance plasmids CJ12 and CJ17 were constructed based on the pbRn6 vector (Horn and Cross, 1997). Plasmid CJ12 was prepared by replacing the reporter cassette of pbRn6 with an inducible T7 promoter-driven GFP cassette using the SpeI and StuI restriction sites. A PCR-amplified blasticidin cassette was introduced downstream of the GFP reporter using the NheI sites generating plasmid CJ11. Next, a rRNA promoter, released from pbRn6 as a SfiI–EcoRI fragment and blunt-ended with T4 DNA polymerase, was inserted in front of the blasticidin cassette using the StuI site. To generate plasmid CJ17, constitutively expressing GFP, the Tet-operator downstream of the T7 promoter was removed by BglII digest of CJ11 followed by religation of the plasmid. Plasmids CJ12 and CJ17 containing GFP under either Tet-inducible or constitutive promoter, respectively, have been electroporated into the 29–13 strain cultured in the SDM-79 medium under standard conditions, and cells resistant to 10 μg/ml of blasticidin have been cloned out. Selected clonal cell lines containing the constitutive GFP vector showed medium to strong fluorescence in about 85% cells, whereas virtually all cells containing the inducible GFP started to strongly fluoresce 12–24 h upon the addition of 1 μg/ml Tet to the medium. Next, 1×10^9 cultured procyclics of both cell lines were mixed with 100 ml of fresh defibrillated inactivated bovine blood and immediately fed through a silicon membrane to teneral or non-teneral flies of both sexes. Engorged flies were placed in cages in small mono-specific and mono-sexed groups of up to 30 specimens and maintained by feeding on fresh defibrillated inactivated bovine blood (first three feedings) and on uninfected rabbits throughout the rest of the experiment (see below). Because of their high permissibility for *T. brucei*, two species of *Glossina* vectors were selected. Populations of *Glossina palpalis gambiensis* and *Glossina morsitans morsitans* were maintained in a level-2 containment insectary (CIRAD, Montpellier, France) at 23 °C and 80% relative humidity. These colonies originate from flies field-collected in Burkina Faso and Zimbabwe, respectively.

Out of a total of 1211 infected tsetse flies, 423 died in the course of the 2-month long experiment, which is in agreement with a mortality predicted from literature for these infected insects (Maudlin et al., 1998). Surviving 788 flies have been microdissected in two different periods. Within 2–15 days post-infection, 41.8% of a total of 287 males and females of teneral *G. p. gambiensis* and *G. m. morsitans* and non-teneral *G. m. morsitans* harboured intestinal infections (Table 1). Both species and sexes have been susceptible for the 29–13 cells, with the infections varying from very strong to weak (Table 1 and Figs. 1 and 2). No significant correlation with the particular host or sex was observed (data not shown). The non-teneral males and females of *G. m. morsitans* were not refractory to the early midgut infection, but the infection rate for non-teneral flies was lower ($p < 0.05$) than in their teneral counterparts (Table 1). As expected, cells containing constructs with constitutively expressed GFP fluoresced in the insect midgut at an equal rate and intensity as in the medium. With the inducible strain, induction was initiated either in parallel with the infection, or during the 2nd or 3rd feeding of infected flies within one week of infection. In all cases the majority of cells started to fluoresce, proving that the induction can be postponed in the course of the intestinal infection.

The next step was to show whether the midgut infection by the *T. brucei* strain 29–13 can progress into the salivary glands and thus complete its life cycle in tsetse. For this, 501 flies of both sexes and species have been dissected and carefully examined 60 days post-infection. Regrettably, none of them harboured trypanosomes in the salivary glands. After two months, the percentage of surviving flies with a late midgut infection dropped to 4.4% (Table 1), however, the persisting infections were high (Fig. 2). At this stage none of the flagellates was found to express GFP at the level visible under fluorescent light. This is not surprising in the case of trypanosomes containing the inducible construct, since they were growing in the absence of Tet for a long period of time. However, the reasons for an eventual shutdown of the constitutive promoter regulating the expression of GFP remain unclear. More than 99% of the non-teneral *G. m. morsitans* flies were able to eliminate the infection during two months, whereas in average 7.5% of infections survived in the teneral males and females of the same species (Table 1). Once the infection is established in a susceptible female fly, it will remain there without shortening the life span of its host. The situation seems to be different for males, where the incubation time in midgut is short, proceeds rapidly into salivary glands, and eventually increases the mortality of the infected specimen (Welburn and Maudlin, 1999; Van den Abbeele et al., 1999). However, our results show that the midgut infection caused by the strain 29–13 does not interfere with the life span of either females or males, and is within two months eliminated probably by the failure of trypanosomes to resist the immune defence of the fly.

Table 1
Intestinal infections of teneral (T) and non-teneral (NT) flies of *Glossina palpalis gambiensis* and *G. morsitans morsitans* in two different periods of dissection

Species	Sex	2–15 DPI		60 DPI	
		Dissected	Infected (%)	Dissected	Infected (%)
G. palpalis T	M	62	26 (41.9)	115	7 (6.1)
	F	69	22 (31.9)	157	9 (5.8)
G. morsitans T	M	25	13 (52.0)	24	2 (8.3)
	F	45	28 (62.2)	43	3 (7.0)
G. morsitans NT	M	42	12 (28.6)	57	1 (1.8)
	F	44	19 (43.2)	105	0 (0)
Total		287	120 (41.8)	501	22 (4.4)

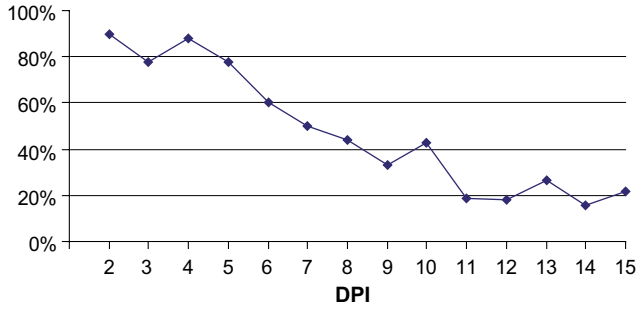

Fig. 1. Percentage of tsetse fly midguts containing trypanosomes within the first 2 weeks after infection.

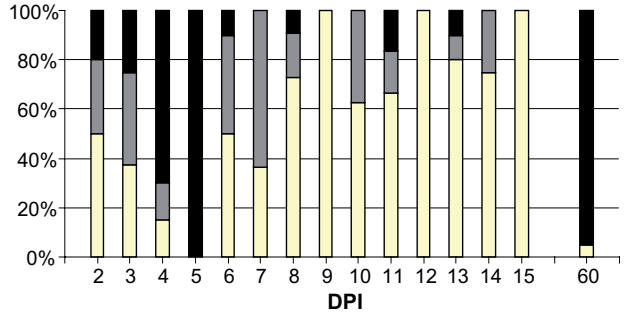

Fig. 2. Intensity of midgut infection within first 2 weeks after infection and 2 months after infection. At least 10 flies were dissected each day. Black bar: heavy infections (>1000 parasites per midgut); grey bar: medium infections (100–1000 parasites per midgut); white bar: light infections (1–100 parasites per midgut).

Usually about 10% of permissive tsetse flies will eventually develop an infection with metacyclic flagellates in the salivary glands, with somewhat higher infection rates being achieved using the bloodstream forms (Van den Abbeele et al., 1999). The sample size used convincingly showed that the strain 29–13 is unable to complete its developmental cycle in the tested *Glossina* spp. vectors. The failure is probably caused by the loss of the ability of trypanosomes to produce salivary gland infections due to either their genetic hypermodification or long passage in the culture media. This explanation seems to be more plausible than a possible refractoriness of tsetse to this strain. As in other protozoan pathogens, repeated transmission of *T. brucei* via its mammalian host will increase its pathogenicity (Hajduk and Vickerman, 1981), whereas an opposing effect of long term cultivation in a simplified environment of the culture medium can be anticipated, as demonstrated for the related flagellate *Leishmania tarentolae* (Thiemann et al., 1994).

Our experiments showed the limits of phenotypic tests of the 29–13 strain in tsetse that can be focused mainly on the early midgut infection. The cells can be induced by RNAi during or shortly after the infection, but they fail to proceed into the salivary glands. The improper development in its insect vector of the most widely used strain and the flagship of RNAi-based functional genomics of *T. brucei* is an impetus to the scientific community to prepare another strain, in which genetically interfered procyclic stages can be studied in their natural environment.

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