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Susceptibility of *Grammomys surdaster* thicket rats to *Trypanosoma brucei gambiense* infection

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Summary Human African Trypanosomiasis is caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense*. Historically, a treatment relapse rate of about 5% is observed in patients treated with melarsoprol, an arsenical derivative used for treatment of both *gambiense* and *rhodesiense* second stage sleeping sickness. More recently, relapse rates up to 30% are noted in *gambiense* sleeping sickness foci in Angola, Sudan and Uganda. Therefore, WHO established a Network on Treatment Failure and Drug Resistance in Sleeping Sickness. One of its objectives is to improve isolation of *T. b. gambiense* from relapsing cases for research on drug resistance mechanisms. *Trypanosoma b. gambiense* isolation techniques suffer from low success rates and long periods needed to adapt the parasite to its new host. Usually, rodents are inoculated with patient's blood or cerebrospinal fluid and sub-passaged until the strain becomes sufficiently adapted to yield high parasitaemia within few days after inoculation. Until now, the best recipient for *T. b. gambiense* is *Mastomys natalensis*, with a success rate of about 50%. In this study, *Grammomys surdaster* (former *Thamnomys surdaster*) was investigated as a potential recipient for isolation of *T. b. gambiense*. Comparative experimental infections of Swiss mice, Wistar rats and *G. surdaster* thicket rats with *T. b. gambiense* clearly show that this trypanosome grows faster in *G. surdaster*. Inoculation of the same rodent species with patient's blood and cerebrospinal fluid in Kinshasa (R.D. Congo) confirms the observation that the thicket rats are more susceptible to *T. b. gambiense* infection than typical laboratory rodents.

keywords *Trypanosoma brucei gambiense*, isolation, rodent, *Grammomys surdaster*

Introduction

Sleeping sickness or human African trypanosomiasis is a lethal disease caused by the protozoan flagellates *Trypanosoma brucei gambiense* and *T. b. rhodesiense* and transmitted by tsetse flies (*Glossina* sp.). The disease occurs in about 35 sub-Saharan African countries with 60 million of people at risk and an estimated 300 000 actually infected persons. The chronic form of sleeping sickness is caused by *T. b. gambiense* and is typically found in West and Central Africa, while a fulminant form is caused by *T. b. rhodesiense* occurring in Eastern Africa (WHO 1998).

Treatment of sleeping sickness patients is difficult. The few drugs that are available have serious adverse effects but as a 'forgotten disease' perspectives for a new generation of less toxic drugs are very poor (Van Nieuwenhove 2000; Legros *et al.* 2002). Historically, a treatment relapse rate of about 5% has been observed in patients treated with melarsoprol, an arsenical derivative used for treatment of both *gambiense* and *rhodesiense* late stage sleeping sickness. More recently, relapse rates of up to 30% have been noted in some *gambiense* sleeping sickness foci in Angola, Sudan and Uganda (Legros *et al.* 2002). Whether all relapsing cases can be attributed to increased resistance of some parasite strains against melarsoprol is a matter of debate but there is no doubt that some *Trypanosoma brucei* strains indeed are more resistant to melarsoprol (Brun *et al.* 2001). Furthermore, resistance to melarsoprol is, at least partly, associated with resistance to pentamidine, the major drug used to treat early stage *gambiense* sleeping sickness and to berenil, a widely used drug against African trypanosomiasis in cattle and small ruminants (Barrett 2001).

Alarmed by this situation, WHO established a Network on Treatment Failure and Drug Resistance in Sleeping Sickness in order to join efforts on drug resistance

surveillance and related research. One of the objectives of this Network is to improve isolation of *T. b. gambiense* strains from treatment relapse cases for drug sensitivity testing and for research on drug resistance mechanisms.

Several techniques for isolation of *T. b. gambiense* from human patients exist but all suffer from low success rates and long adaptation periods needed to propagate the parasite in sufficient numbers (Babiker & Le Ray 1981). Isolation techniques that result in a procyclic population are less useful since drug sensitivity testing should be carried out on bloodstream forms. Usually, such bloodstream populations are obtained by inoculating rodents with the patient's blood or cerebrospinal fluid and by sub-passaging in the same or other rodent species until the strain becomes sufficiently adapted to yield high parasitaemia within few days after inoculation (Mehlitz 1978). Ideally, the number of passages through laboratory animals should be kept as low as possible before the first drug sensitivity testing is started. Until now, the best recipient for isolation of *T. b. gambiense* is the *Mastomys natalensis* rat still with a success rate of only 50% when inoculated with 500–1000 trypanosomes compared with 30% success rate in suckling rats (Mehlitz 1978; Zillmann *et al.* 1984).

Here we report on the apparent high susceptibility of another rodent species for *T. b. gambiense* infection. *Grammomys surdaster* (former *Thamnomys surdaster*) is a 10 cm long thicket rat belonging to the family of *Muridae*. The species lives in and around trees in mountain forests of Central Africa and is the natural mammalian host of *Plasmodium berghei*. Already in 1962, a breeding colony with animals captured in what is now R.D. Congo, was established for studies on *Plasmodium*, *Leishmania*, *Trypanosoma* and other parasites (Yoeli *et al.* 1963). In 1976, Frézil and Carnevale reported on the infectivity of *T. b. gambiense* for *Thamnomys* (without mentioning the species) (Frézil & Carnevale 1976). At our institute, a colony of *G. surdaster* was kept for research on *Plasmodium berghei* (Chatterjee *et al.* 2001). In the present study, *G. surdaster*, Wistar rats and white mice were inoculated with a laboratory kept *T. b. gambiense* strain and with *T. b. gambiense* containing blood and cerebrospinal fluid of human patients to compare the rodents' suceptibility to infection.

Materials and methods

Grammomys surdaster

About 50 animals were trapped around Kaindu near the Kafua National Park (Zambia) in 1996. Determination of the species was based on analysis of the cytochrome B sequence of mitochondrial DNA (Chatterjee *et al.* 2001). Since their arrival at the Institute of Tropical Medicine in Antwerp they mated and reproduced well in captivity. In June 2002, 20 couples were sent to Kinshasa for setting up a breeding colony which currently provides sufficient youngsters for inoculation experiments with sleeping sickness patients' blood and cerebrospinal fluid. The animals are kept on a diet of normal laboratory rodent pellets, supplemented with apple and carrot. *Grammomys surdaster* thicket rats are more nervous than laboratory rodents and some experience is needed to manipulate them correctly. Ambient temperature should not exceed 25 °C. Details on breeding *G. surdaster* thicket rats can be found in Yoeli *et al.* (1963). For the experiments, 15-week-old animals weighing about 30 g were used.

Mice and rats

Female mice [Ico: OF1(Caw) from Charles River Laboratories, Belgium] of about 30 g and male rats (Wistar Unilever HsdCpb:WU from Harlan, The Netherlands) of about 400 g were purchased from Harlan Netherlands for the experiments in Antwerp. Male and female NMRI mice of about 30 g and male and female Wistar rats of about 300 g from the local breeding colony at INRB were used for the experiments in Kinshasa.

Trypanosomes

Experiments in Antwerp. A slow growing clone was chosen from the cryobank collection in liquid nitrogen. *Trypanosoma brucei gambiense* AnTat 11.20, stabilate ITMAS 261191, is a bloodstream form population originally isolated from a child in Zaire in 1974 and identified as *T. b. gambiense* MBA Kinkole/74/ITMAP/1811. The strain was isolated through inoculation of 1 ml of the patient's blood into a female NMRI mouse treated with cyclophosphamide. It was further adapted to rat and mouse by several passages for cloning and serotyping (Babiker & Le Ray 1981). The strain has been identified as *T. b. gambiense* Type I in PCR (Radwanska *et al.* 2002). The AnTat 11.20 clone induces relapsing infection in Wistar rat. The stabilate was thawed in a water bath at 37 °C, diluted twice with phosphate buffer supplemented with glucose (PSG) (Lanham & Godfrey 1970) and checked for viability before intraperitoneal injection of 0.5 ml in a mouse for prior expansion of the population. At first peak parasitaemia (10 days after inoculation), infected blood was collected by cardiac puncture on an heparinized syringe, parasitaemia was estimated by the Matching Method (Herbert & Lumsden 1976) and blood was diluted in PSG to 1 trypanosome per microscopic field

at a 10 × 40 magnification which corresponds with $10^{6.3}$ trypanosomes/ml.

Experiments in Kinshasa. Blood on heparin and plain cerebrospinal fluid (CSF) was collected from consenting sleeping sickness patients presenting at the diagnostic facility of the Programme National de Lutte contre la Trypanosomiase Humaine Africaine (PNLTHA) in Kinshasa. The samples were checked for presence of trypanosomes (mAECT for blood, double centrifugation for CSF) and were transported on ice to the INRB for inoculation within 2 h. In total, seven blood samples and seven CSF samples from different patients were used. Since the patients were most probably infected in Bandundu and Kinshasa Province, we consider the trypanosomes as putative *T. b. gambiense* type I.

Infection and follow-up

Experiments in Antwerp. Ten animals of each rodent species were inoculated intraperitoneally (IP) with a suspension of 1 trypanosome per microscopic field at 10 × 40 magnification. *Grammomys surdaster* and OF-1 mice received 0.3 ml. Wistar rats received 3 ml. Parasitaemia was checked at regular intervals after inoculation during 14 days. Five µl of tail vein blood were dispensed on a microscope slide and covered with a 24 × 24 mm coverslip. Depending on the parasitaemia, the number of trypanosomes in 1 up to 100 microscopic fields at 10 × 40 magnification was counted.

Experiments in Kinshasa. Each blood or CSF sample was inoculated intraperitoneally into three animals of each rodent species (*G. surdaster* thicket rat, NMRI mouse, Wistar rat). *G. surdaster* thicket rats and NMRI mice received 0.3 ml, Wistar rats received 0.5 ml. At the moment of inoculation, the number of parasites in blood was estimated between 180 000 and 240 000/ml while in CSF this number varied between 60 000 and 120 000/ml. Parasitaemia was checked at regular intervals similar to the experiments in Antwerp up to 50 days post-inoculation (DPI). All animals surviving the experiment observation period were killed.

Ethical clearance

For the use of laboratory animals, ethical clearance was obtained from the Veterinary Ethical Committee of the Institute of Tropical Medicine in Antwerp. For the use of patients' biological samples, the study received ethical clearance from the Ethical Committee of the Ministry of Public Health in R.D. Congo.

Results

Comparative susceptibility of the three rodent species to infection of the *T. b. gambiense* AnTat 11.20 clone

Tables 1–3 represent observed parasitaemia in the individual animals of each species in function of time after inoculation. Figure 1 represents the first peak parasitaemia for each rodent species as box plots. Individual variability is highest within the *G. surdaster* group. First peak parasitaemia occurred between 3 and 10 DPI (median 6) and ranged between 2300 and 40 000 trypanosomes/100 fields with a median value of 27 000 trypanosomes/100 fields (corresponding to

Table 1 Parasitaemia (trypanosomes/100 fields) in *Grammomys surdaster* inoculated IP with 0.3 ml at 1 trypanosomes/field

Animal number	Days after infection					
	3	5	7	10	12	14
1	12 000	**40 000**	*			
2	0	470	**2300**	1700	36	1650
3	2	68	**30 000**	5400	40 000	40 000
4	7500	**24 000**	*			
5	0	16	25 000	**40 000**	*	
6	**20 000**	15 000	*			
7	**10 800**	6300	*			
8	18 000	**30 000**	*			
9	4	4500	**40 000**			
10	0	3	10 500	**16 600**	*	

First peak parasitaemia values are in bold; * dead.
One trypanosome/100 fields corresponds to $10^{4.3}$ trypanosomes/ml.

Table 2 Parasitaemia (trypanosomes/100 fields) in OF-1 mice inoculated IP with 0.3 ml at 1 trypanosomes/field

Animal number	Days after infection					
	3	5	7	10	12	14
1	2700	**7200**	98	12	1800	1430
2	0	1	**1300**	0	0	0
3	**3600**	8	0	1	210	2700
4	4400	**5600**	255	3	16	74
5	3800	25000	**40 000**	27 000	28 000	14 000
6	**1900**	1600	0	3000	21 000	5800
7	**2200**	0	159	0	40	1150
8	**2300**	2100	213	0	12	2300
9	**4800**	4000	0	4	320	650
10	2300	**8200**	220	6	18	3700

First peak parasitaemia values are in bold.
One trypanosome/100 fields corresponds to $10^{4.3}$ trypanosomes/ml.

Table 3 Parasitaemia (trypanosomes/100 fields) in Wistar rats inoculated IP with 3 ml at 1 trypanosomes/field

Animal number	Days after infection					
	3	5	7	10	12	14
1	23	9	**250**	5	1500	570
2	22	**1300**	310	0	2000	216
3	32	1800	**6400**	1	48	1500
4	93	12	**960**	0	490	2700
5	97	9	**500**	0	650	0
6	56	**1900**	600	51	1900	1800
7	**75**	0	280	1	80	0
8	110	124	**750**	3	360	380
9	67	**2400**	600	4	140	310
10	78	6	**340**	2	410	27

First peak parasitaemia values are in bold.
One trypanosome/100 fields corresponds to $10^{4.3}$ trypanosomes/ml.

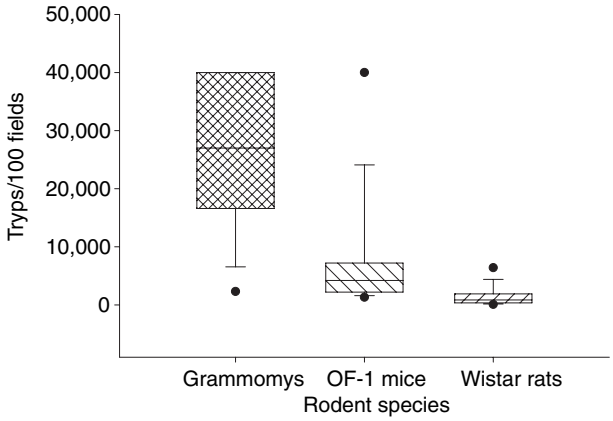

Figure 1 Box plot representation of first peak parasitaemia in different rodent species (10 animals each) inoculated with *T.b. gambiense* AnTat 11.20. Solid line within box: median. Lower and upper edge of box: 25th and 75th percentile. Lower and upper whisker: 10th and 90th percentile. Black dots: individual outliers.

$2.7 \times 10^{8.3}$ trypanosomes/ml). Half of the animals did not survive the first peak parasitaemia. In OF-1 mice and rats, the individual variability is lower than in *G. surdaster*. In mice, the first peak parasitaemia occurred within 7 days (median 4) and ranged between 1300 and 40 000 trypanosomes/100 fields (median 4200 trypanosomes/100 fields). In rats the first peak parasitaemia occurred within 7 days (median 7) and ranged between 75 and 6400 trypanosomes/100 fields (median 855 trypanosomes/100 fields). All OF-1 mice and Wistar rats survived the first peak parasitaemia.

Comparative susceptibility of the three rodent species to infection with wild type *T. b. gambiense* strains

From the seven blood samples inoculated, one did not initiate any detectable infection in the three rodent species (Table 4). From the other six samples, only three of 18 Wistar rats and one of 18 NMRI mice showed a detectable infection within 50 days while 16 of 18 *G. surdaster* thicket rats showed detectable infection within this observation period. The median value for appearance of first parasitaemia calculated on the positive *G. surdaster* is 13 DPI with maximum parasitaemia up to 3 trypanosomes/100 fields. Second peak parasitaemia was generally higher with up to 73 trypanosomes/100 fields in one animal at 15 DPI (data not shown).

From the seven CSF samples inoculated, three did not lead to any detectable infection in the three rodent species (Table 5). With one sample, all nine animals became

Table 4 First day of detectable parasitaemia in *G. surdaster* thicket rats, Wistar rats and NMRI mice inoculated with patients' blood

Species	Number	Patient number						
		1	2	3	4	5	6	7
Grammomys	1	16	*n*	16	5	9	5	5
	2	*n*	*n*	34	5	5	16	16
	3	14	*n*	7	5	*n*	5	22
Wistar	1	*n*	*n*	*n*	27	*n*	*n*	23
	2	*n*	*n*	*n*	37	*n*	*n*	*n*
	3	*n*	*n*	*n*	*n*	*n*	*n*	25
NMRI	1	*n*	*n*	*n*	45	*n*	*n*	*n*
	2	*n*	*n*	*n*	*n*	*n*	*n*	*n*
	3	*n*	*n*	*n*	*n*	*n*	*n*	*n*

n, remained negative during 50 days follow-up period.

Table 5 First day of detectable parasitaemia in *G. surdaster* thicket rats, Wistar rats and NMRI mice inoculated with patients' CSF

Species	Number	Patient number						
		1	2	3	4	5	6	7
Grammomys	1	15	7	40	8	*n*	*n*	*n*
	2	7	7	37	10	*n*	*n*	*n*
	3	*n*	7	29	*n*	*n*	*n*	*n*
Wistar	1	*n*	7	*n*	*n*	*n*	*n*	*n*
	2	*n*	17	*n*	*n*	*n*	*n*	*n*
	3	*n*	7	*n*	*n*	*n*	*n*	*n*
NMRI	1	*n*	6	*n*	*n*	*n*	*n*	*n*
	2	*n*	7	*n*	*n*	*n*	*n*	*n*
	3	*n*	7	*n*	*n*	*n*	*n*	*n*

n, remained negative during 50 days follow-up period.

infected while with the other three samples seven of nine *G. surdaster* thicket rats and none of the Wistar rats nor of the NMRI mice showed detectable infection within the 50-day observation period. The median value for appearance of first parasitaemia calculated on the positive *Grammomys* was 9 DPI. First peak parasitaemia in these animals was low (up to 3 trypanosomes/100 fields) but maximum second peak parasitaemia went up to 800 trypanosomes/100 fields (data not shown). In addition, from the infected *G. surdaster*, we prepared cryostabilates originating from four blood and three CSF samples which all proved to be viable after 1 week storage in liquid nitrogen.

Discussion

This study was set up to investigate whether the thicket rat, *G. surdaster*, could be a more susceptible rodent for isolation of *T. b. gambiense* from sleeping sickness patients, compared with classical laboratory rodents such as Wistar rats or OF-1 and NMRI mice. For practical reasons, a comparison with *Mastomys natalensis* was not planned. The first experiment with a slow growing *T. b. gambiense* clone showed that indeed *T. b. gambiense* grows faster and reaches much higher parasitaemia in *G. surdaster*. However, individual variability is higher than observed in Wistar rats and OF-1 mice.

Keeping in mind that the *T. b. gambiense* AnTat 11.20 clone strain was already adapted to laboratory rodents and that an inoculum of 0.3–0.5 ml with 1 trypanosome per microscopic field is still high compared with the numbers of trypanosomes in patients' blood or CSF, the findings of this experiment had to be confirmed with wild type trypanosome strains in patients' samples. Therefore, the animal house at INRB in Kinshasa was rehabilitated, the Wistar rat colony was provided with young male rats for breeding and a *G. surdaster* colony was started. Within a few months, sufficient NMRI mice, Wistar rats and *G. surdaster* thicket rats were available for further inoculation experiments. After obtaining approval of the National Ethical Committee and after arrangements with the PNLTHA, three animals of each rodent species were inoculated with blood or CSF of non-treated consenting patients where after the animals were checked for patent parasitaemia during almost 2 months.

This experiment clearly confirmed the findings with the *T. b. gambiense* AnTat 11.20 clone. Although some of the inoculations, with blood as well as with CSF, did not lead to detectable infections, the *G. surdaster* thicket rats seem easier to infect than the Wistar rats or the NMRI mice. Most animals of both latter species remained negative while 10 of 21 *G. surdaster* became positive with CSF and 16 of 21 with blood resulting in successful isolation of *T. b. gambiense* from four of the seven CSF samples and from six of the seven blood samples. These isolation success rates are higher than the 50% success rate observed in *Mastomys natalensis* (Mehlitz 1978). As expected, some variability between individual animals was observed: first detection of trypanosomes occurred between 5 and 40 days. First peak parasitaemia remained low but second peak parasitaemia and first peak parasitaemia after one sub-inoculation were higher and sufficient to prepare stabilates in liquid nitrogen.

Taking these findings into account, for improved isolation of *T. b. gambiense* through *G. surdaster* thicket rats, it is proposed to inoculate at least two animals and to sub-inoculate them once or twice within a few days in new animals, even without confirmation of infection. Thus the appearance of antibodies which otherwise may destroy the first peak population, of which the occurrence cannot be precisely predicted, can be avoided resulting in parasitaemia high enough to cryopreserve the isolate.

Hitherto, the isolation of *T. b. gambiense* from CSF directly into laboratory rodents has been found very difficult. The fact that trypanosomes in CSF also easily infect *G. surdaster* is particularly interesting since most of the patients refractory to treatment are diagnosed with trypanosomes in the CSF, not in the blood.

Other recipient rodents with putative higher susceptibility, such as SCID mice or immunosuppressed mice and rats, could be considered for improved isolation of *T. b. gambiense*. However, genetically or induced immunodeficient animals are more vulnerable to other infections or may be difficult to propagate. Furthermore, in such animals higher parasitaemia may be reached because of the absence of an immune response but this does not guarantee that prepatent times will be shorter than in *G. surdaster*.

Our experience with the *G. surdaster* thicket rats is that they are easy to breed and that only little experience is needed to handle them properly. We therefore started to use them for the isolation of trypanosomes from treatment refractory patients in R.D. Congo and for obtaining cryostabilate populations available for research on the mechanisms of drug resistance.

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