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Monitoring the pleomorphism of *Trypanosoma brucei gambiense* isolates in mouse: Impact on its transmissibility to *Glossina palpalis gambiensis*

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ABSTRACT

Substantial differences have been observed between the cyclical transmission of three *Trypanosoma brucei gambiense* field isolates in *Glossina palpalis gambiensis* (Ravel et al., 2006). Differences in the pleomorphism of these isolates in rodent used to provide the infective feed to *Glossina*, could explain such results, since stumpy forms are preadapted for differentiation to procyclic forms when taken up in a tsetse bloodmeal. To assess this possibility, mice were immunosuppressed and inoculated intraperitoneally with the three isolates (six mice for each trypanosome isolate); then parasitaemia and pleomorphism were determined daily for each mouse. The three *T. b. gambiense* isolates induced different infection patterns in mouse. The parasitaemia peak was rapidly reached for all the isolates and maintained until mice death for two isolates, while the third isolate rapidly showed a falling phase followed by a second parasitaemia plateau. The proportion of the stumpy forms varied from 15% to 70% over the duration of the experiment and according to the isolate. One isolate, which displayed the highest proportion of stumpy forms and reached the stumpy peak at the onset of the falling phase of parasitaemia, was used to study the relationship between the proportion of stumpy forms and transmissibility to tsetse fly. The results indicated that the transmissibility of trypanosomes was not correlated to the proportion of non-dividing stumpy forms.

1. Introduction

Human African trypanosomiasis (HAT) is caused by trypanosomes belonging to *Trypanosoma brucei* species, transmitted to humans by tsetse flies. *Trypanosoma brucei gambiense* (*T. b. gambiense*) is responsible for the chronic form of HAT in West and Central Africa where the main vectors are *Glossina* species of the *palpalis* group (Hoare, 1972). The ability to transmit *T. b. gambiense* requires the completion of a complex life cycle by the parasite in the tsetse fly: establishment of the procyclic forms of the parasite in the midgut (immature infections) followed by the maturation of the parasites into metacyclic forms in the salivary gland (mature infections) (Vickerman, 1985; Welburn and Maudlin, 1999). The epidemiology of HAT is therefore determined to a large extent by the number of tsetse flies with mature infections in a specific area.

Previous study of the cyclical transmission of *T. b. gambiense* in *Glossina palpalis gambiensis* (*G. p. gambiensis*), showed substantial differences between different field isolates originated from the same sleeping sickness focus (Ravel et al., 2006). Whereas some isolates displayed only immature infections, one isolate gave both immature and mature infections and one led only to mature infections. Because such differences could have important implications for the epidemiology of the transmission of HAT, it is essential to determine the reasons for these differences. The trypanosome stock can play a role (Maudlin and Welburn, 1994) as can numerous factors such as the pleomorphism of the parasites. In the mammalian bloodstream, the parasite population is described as pleomorphic with slender and stumpy forms as well as transitional forms between the slender and stumpy forms defined as intermediate forms (Vickerman, 1965; Matthews et al., 2004). Trypanosomes first divide as long-slender bloodstream forms. At a certain threshold density, the trypanosomes differentiate to cell cycle-arrested short stumpy blood forms (Reuner et al., 1997; Vassella et al., 1997). This differentiation and the distinct biology of the slender and stumpy forms enable the trypanosome to fulfil its dual objectives in the mammalian host: to proliferate and to

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ensure transmission to tsetse fly (Matthews, 1999). The slender form parasites are adapted to exploiting the rich glucose environment of the blood (Bakker et al., 1995) and they undergo rapid multiplication, allowing them to establish the parasitaemia in the mammalian host. In contrast, the stumpy forms do not divide and there are changes in their enzyme and mitochondrial functions (Tyler et al., 1997) as they prepare for survival in the glucose-poor, poorly oxygenated environment of the tsetse gut. Stumpy forms thus appeared to be vector-transmissible (Seed and Wenck, 2003).

The aim of the present study was first to monitor the pleomorphism of different isolates of *T. b. gambiense* during their development in mice and then to study the impact of this pleomorphism on the parasite transmissibility to *G. p. gambiensis*.

2. Materials and methods

2.1. Trypanosomes

The three *T. b. gambiense* isolates used here (S1/1/6, S7/2/2 and S12/9/5) were isolated in 2002 by rodent inoculation from three HAT patients detected in the sleeping sickness focus of Bonon, Côte d'Ivoire. They all belong to *T. b. gambiense* group 1. One cryostabilate of each isolate, resulting from five previous passages in mice, was reactivated in two BALB/c mice. Then one more passage for multiplication was done before inoculation of six mice. Finally, each isolate was passaged only seven times in rodents since its isolation from the field, ensuring the pleomorphism of the bloodstream populations. Indeed, it has been demonstrated that following long-term passage in laboratory animals, trypanosome lines have been selected that no longer generate stumpy forms in rodent infections (Ashcroft, 1960).

2.2. Tsetse flies

Male flies from the *G. p. gambiensis* in vivo colony maintained at CIRAD-UMR Trypanosomes in Montpellier, France were used throughout the study. This colony originated from Burkina Faso. Only male flies were used because males infected with trypanosomes of the *Trypanozoon* subgenus develop a greater proportion of salivary gland infections than females of the same species (Maudlin et al., 1991; Dale et al., 1995; Milligan et al., 1995).

2.3. Monitoring the development of the parasites

Eighteen BALB/c mice (six for each trypanosome isolate) previously immune-suppressed with cyclophosphamide (Endoxan®, 300 mg/kg), were injected intraperitoneally with 0.3 ml of blood containing 8×10^6 trypanosomes per millilitre. Immunosuppression was maintained using cyclophosphamide every 5 days. The parasitaemia of each mouse was measured daily using the matching method (Herbert and Lumsden, 1976), beginning the third day after infection for 30 days, and thereafter 2 days a week until death. For each mouse the relative proportion of stumpy, slender and intermediate forms was determined as follows. Thin films of tail blood were obtained daily on glass slides and then trypanosomes were stained using the RAL 555 kit (Reactifs RAL, Martillac, France), a fast-acting variation of May–Grunwald Giemsa staining. One hundred trypanosomes in consecutive microscope fields of the blood film were counted (1000× magnification) and identified as stumpy, slender or intermediate according to Hoare (1972) (see also Fig. 1). For analysis, we considered intermediate forms, which will transform to stumpy forms, together with stumpy forms, in opposition to slender forms. Pleomorphism could not be determined for parasitaemia lower than 16×10^6 tryps/ml because the number of parasites was too low to count. Since one mouse injected with S12/9/5 died the day after injection, only five mice were monitored for this isolate.

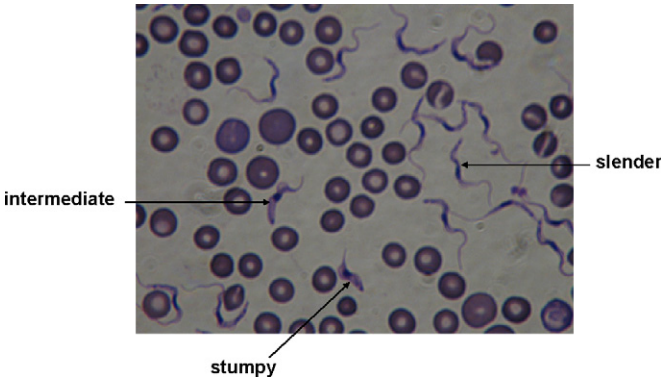

Fig. 1. The pleomorphism of *T. b. gambiense* isolate S7.2.2 in mouse. Blood smear from the S7/2/2-infected BALB/c mouse was stained with a fast-acting variation of May–Grunwald Giemsa and observed by microscopy at 1000× magnification. Slender, intermediate and stumpy forms are indicated by arrows.

2.4. Infection of tsetse flies

Six BALB/c mice, previously immune-suppressed with cyclophosphamide (Endoxan®, 300 mg/kg), were injected intraperitoneally with 0.3 ml of blood containing 3.2×10^7 tryps/ml of the S7/2/2 stabilate. From the second day after the infection onwards, parasitaemia and pleomorphism of trypanosomes of each mouse were measured daily as described above. A first batch of teneral flies (78 for batch no. 1 and 102 for batch no. 1′) was fed on the bellies of anaesthetized infected mice showing rare stumpy + intermediate forms while a second batch (84 for batch no. 2 and 123 for batch no. 2′) was fed on the bellies of anaesthetized infected mice showing maximum of stumpy + intermediate forms. Mice were anaesthetized with 0.2 ml per mouse of a mixture of Ketamine® (Merial) and Rompun® 2% (Bayer). After feeding, all the flies were retained for the experiment because we had previously observed that flies with no obvious feeding were positive for trypanosomes during dissection (unpublished data). Then all the flies were maintained by feeding on a rabbit, 3 days a week, until the end of the experiment. To reduce the risk of re-infecting the flies, before each meal, the rabbit was monitored for parasites by examination of the buffy-coat (Murray et al., 1977) prepared from a blood sample taken from the ear, using phase contrast microscopy at 400× magnification.

2.5. Dissection of tsetse flies

On the 45th day after the infective feed, the flies which were still alive were dissected according to the method described by Penchenier and Itard (1981). The time lag between infection and dissection was sufficient for the completion of the *T. b. gambiense* developmental cycle in tsetse (Van Den Abbeele et al., 1999). Before dissection, the flies were starved for 72 h to reduce partially digested blood and thus facilitate observation of the trypanosomes in the midguts. Midgut and salivary glands were examined for trypanosomes by phase contrast microscopy at 400× magnification.

3. Results

3.1. Monitoring the parasitaemia and the pleomorphism of the three *T. b. gambiense* isolates

Each batch of six mice injected with the same isolate showed quite similar development of trypanosomes (Fig. 2). To compare