Clinical Follow-Up in the Rat Experimental Model of African-Trypanosomiasis

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Animal models of Human African Trypanosomiasis (HAT) have been developed to understand the pathogenic mechanisms leading to the passage into the neurological phase, most of them referring to histological aspects but not clinical or behavioral data. Our study aimed at defining simple clinical and/or behavioral markers of the passage between the hemolymphatic phase and the meningo-encephalitic stage of the disease. Sprague-Dawley rats (n = 24) were infected with *Trypanosoma brucei brucei* AnTat 1.1E. Food intake and body weight were measured daily from the day of infection until death. Hematocrit was measured twice a week. Behavioral disturbances were evaluated through an Open-field test. A sudden weight loss occurred on the twelfth day after infection, due to a significant drop of food intake starting two days before. The rats developed an anemic state shown by the hematocrit measurements. The Open-field test showed them to be less active and reactive as soon as the second week after infestation. A complementary histological study observed trypanosomes and inflammatory cells in the choroid plexus at the same period. These results are in favor of central nervous system functional disturbances. The observed weight loss is discussed as being a parameter of the entry in the meningo-encephalitic phase. The rat model reproduces neurological symptoms observed in the human disease and may prove to be useful for further neurohistological and therapeutic studies. Exp Biol Med 228:1355–1362, 2003

Key words: experimental African trypanosomiasis; rat model; food consumption; body weight; open field

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Human African Trypanosomiasis (HAT) or sleeping sickness is caused by the inoculation of a flagellate protozoa *Trypanosoma brucei gambiense* (in Western and Central Africa) or *T. brucei rhodesiense* (in Eastern Africa) by tsetse flies or *Glossina* (1–4). An estimated 50 million African residents are at risk of being infected and, according to the World Health Organization, sleeping sickness is still among the infectious "leading killers" (5).

In the first hemolymphatic stage of the disease, the parasites invade the blood and the lymphatic system. The most common signs are intermittent fever, headache, joint pain, edema, and lymphadenopathies. Irregular febrile episodes are accompanied by headache, malaise, and anemia. At the second stage, trypanosomes penetrate the central nervous system (CNS) and can be found in the cerebrospinal fluid (CSF). The terminal stage of the disease is characterized by the irreversibility of the lesional process with demyelinating encephalitis. During the meningo-encephalitic stage, varied neurological symptoms worsen progressively. They can be summarized as follows: daytime somnolence, intermittent fever, headaches, sensory disturbances with uncomfortable diffuse superficial or deep sensations (hyperpathia), presence of primitive reflexes (palmo-mental reflex, sucking reflex), exaggerated deep tendon reflexes, pruritus, with or without skin lesions, and tremor (fine and diffuse without any myoclonic jerk at rest or during movement). Psychiatric disorders are frequent, with mood swings, confusion, agitation, aggressive behavior, and euphoria, absent gaze, mutism, indifference, and stereotypic behavior and epileptic seizures. The spontaneous evolution of the disease leads to death in a dramatic cachectic state if untreated (6–8).

Although the pathological reactions of the host have been extensively investigated in both humans and experimental animals, the correlation between anatomical changes and clinical symptoms and the pathogenic mechanisms behind the clinical and pathological alterations still remain to

be clarified. Also, drugs active at the meningo-encephalitic phase cross the blood–brain barrier, but are highly toxic, which justifies the development of animal models of HAT. However, until now, the golden standard to assess the stage of the disease is the neuropathologic examination. It is therefore necessary to develop a model in which the diagnosis of the stage of evolution of the disease can be made simply.

Different animal models have been proposed, such as trypanosome-infected mouse, rat, dog, sheep, and monkey (9), using three pathogenic strains (human: *T. b. gambiense* and *T. b. rhodesiense*; rodents: *T. b. brucei*). Animal models of chronic infection were used at the neurological phase of the disease to study the *in vivo* efficacy of drugs known to be active on trypanosomes *in vitro* (10). However, physiological and clinical data have been seldom reported in rodent models. This may be because most of the studies were performed on larger animals, as they focused on the immunological reaction to the invading parasites (11) and/or the pathogenesis of the CNS alterations. Available physiological data (e.g., body temperature, motor activity, food intake, etc.) describing the clinical state of the animals during the time course of the disease are therefore scarce (12).

The present study was therefore carried out aiming at a better understanding of the time course of the clinical symptoms of the disease in a *T. b. brucei*-infected rat model. Such a model could then be used as an alternative to pathological examination for drug screening for instance.

Materials and Methods

The experimental protocol received the agreement of the institutional ethics committee for animal care and the use of animals for research purposes.

Animals. The experiment was carried out in male Sprague-Dawley rats (IFFA-CREDO, Lyon, France) with an initial weight of 200 to 230 g. They were housed in individual sound-attenuated Plexiglas cages and maintained under standard laboratory conditions: 21 to 22°C ambient temperature, food and water available *ad libitum*, and a standard 12:12-h light:dark cycle with lights on at 2:00 PM and lights out at 2:00 AM. They were randomly assigned to two groups, one of which was used as control ($n = 12$) and the other ($n = 24$) was infected.

Trypanosomes and Infection Procedure. *Trypanosoma brucei brucei* AnTat 1.1E, a pleiomorphic clone derived from an EATRO cryostabilate (East African trypanosomiasis research organization) 1125, isolated in 1966 from blood of the *Tralephagus scriptus* in Uganda and kindly provided from the Prince Leopold Institute of Tropical Medicine at Antwerp, Belgium, was used as previously described (13, 14).

Briefly, trypanosomes were re-warmed by mixing the cryostabilate in 0.9 NaCl containing 1% glucose (1:5 ratio) before use. Mobility of trypanosomes was controlled with an optic microscope and the density was adjusted to 10^4 per mL. Twenty-four rats were infected (day D_0) by intraperitoneal injection of 200 µl of the above solution containing approximately 3000 trypanosomes. Parasitemia was checked regularly by direct microscope observation of a wet blood film from a tail sample.

Measurements. Body weight was measured each morning at the same time of day. All rats were fed the appropriate diets from metal cups specifically designed to minimize food spillage. The daily food intake of each rat was determined directly by weighing the cup just before and after refilling.

Every Monday and Thursday, a blood sample was withdrawn between 9:00 and 12:00 AM to measure the hematocrit. The animals were maintained for a few seconds in an appropriate cage flowed with a 4% halothane (Laboratoire Belamont, Paris, France) to 96% oxygen mixture; the tip of the tail was cut with a scalpel and blood (~50 µl) was directly collected into a specific hematocrit-measuring tube (Haematokrit Kapillaren, Hirschmann Laborgerate, Eberstadt, Germany). The tube was then centrifuged 5 min at 12,000 revs per minute and the hematocrit measured using a manual reading plate. After the blood sampling, the wound was cleaned up with betadine® (Laboratoire ASTA Médica, Mérignac, France) and tapped with coalgan® (Les Laboratoires Brothier S.A., Nanterre, France).

Open-Field Tests. The animals were submitted to an Open-field test at D_3, D_8, D_{15}, D_{22}, D_{29}, D_{36}, and D_{42}. This test allows the evaluation of the motor behavior and the psychomotor capacity (reactivity) of the animal. Animals were tested in a waterproof, painted, wooden open-field box (1 × 1 m) bordered by 15-cm-high walls. The Open-field was illuminated by normal fluorescent room light (~150 lux). Throughout the test, the path of the rat was recorded by a homemade computerized image analysis system (accuracy <1 cm in distance and one-tenth of second in time).

To test the animals as quickly as possible to limit the time-lapse between the first and last tested animals, six rats (two control rats and four infected rats) were tested between 10:00 and 12:00 AM during the light-out activity phase and six rats were tested between 3:00 and 5:00 PM during the light-on resting period.

The Open field was divided into three zones: central (20 × 20 cm), intermediate, and peripheral. The peripheral zone includes the 10-cm-wide strip along the walls, the corners (10 × 10 cm), and the walls themselves. At the beginning of the test, the rat was placed exactly on the center of the Open field. Its behavior was observed thereafter and spontaneous motor activity was recorded for 10 min. Before introducing an animal on the Open field, the arena was cleaned with 5% alcohol to eliminate odors from the preceeding rat.

The following measures were performed: total length of the rat itinerary (in m), latency to leave the center (in s), and time spent in the intermediate, central, and peripheral zones.

Histology. To complete information on the staging of HAT by reference to clinical and behavioral criteria, we

conducted a histological study that provides correlative information on whether the trypanosome has entered the central nervous system (CNS).

Six male Sprague Dawley rats (200 to 250 g) were infected by intraperitoneal injection of approximately 3000 trypanosomes in 200 µl of a solution NaCl 0.9% containing 1% glucose, as previously described. Infected rats were randomly sacrificed on D_{13} and D_{15} and encephala removed for histological examinations.

After ether anesthesia, rats received intracardiac perfusion with 4% paraformaldehyde aqueous solution. The fixed encephala were removed and suffixed in the same solution for 48 hrs. The fixed samples were embedded in paraffin. Sections of 3 to 5 µm in thickness were cut with a microtome (Reichert-Jung, Biocut) and stained with hematoxylin and eosin.

Statistical Analysis. Calculations were performed using the StatView package (SAS Institute Inc, Cary, NC). A two-way ANOVA with time and group effects was used, followed by between-group comparisons using the Bonferroni correction. Student's *t* test was used for the comparison of Open-field data obtained during the activity and resting phases. All the data are presented as the mean ± standard deviation; statistical significance was set at $P < 0.05$.

Results

None of the controlled rats did die during the experiment. Infected rats died in 15.3 ± 12.4 days, death occurring between the tenth and forty-sixth days of infection, time of death following a gaussian distribution (Fig. 1).

Parasitemia. Trypanosomes were observed in the blood 5 days after inoculation (Fig. 2).

Food Intake and Body Weight. Daily food consumption equalled 30.84 ± 2.22 g in control rats and 29.98 ± 6.11 g until D_{11} in infected rats. In the latter animals, it then decreased ($P < 0.05$) (Fig. 3, Fig. 4). In all infected rats, weight loss followed the decrease in food consumption.

In control rats, body weight increased steadily throughout the experiment (Fig. 3A) with a mean daily gain of about 4.06 ± 1.23 g. The body weight curve of infected rats followed a similar curve during the first days after infection.

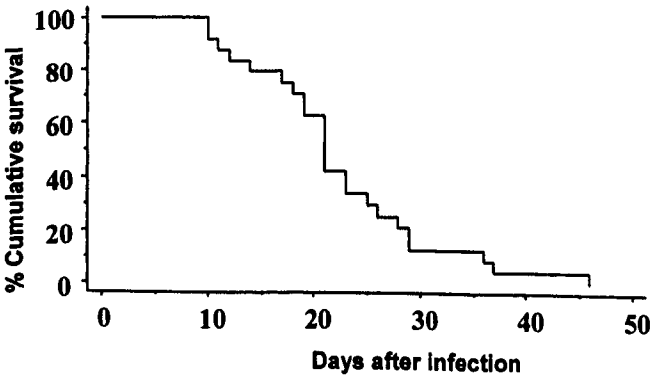

Figure 1. Kaplan-Meier Graph of cumulative survival of 24 rats after infection with *T. brucei brucei* AnTat 1.1E.

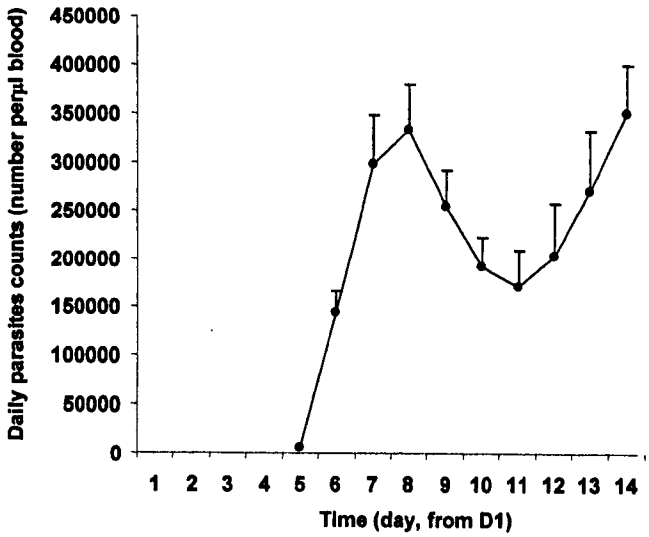

Figure 2. Daily parasite counts expressed as number of trypanosomes present per µl of blood. Data points represent the mean ± SD.

However, the weight curve started to level off, then decrease (Fig. 3B), leading to a significant difference between the two rats groups at D_{14} ($P < 0.034$, Fig. 3A).

Sixteen of the 24 infected rats showed a characteristic weight curve, with a sharp and large drop (Fig. 3B) occurring 13.6 ± 4.7 days after infection. In those rats, daily weight loss was then constant until death occurred 8.7 ± 4.3 days later. Three of the eight remaining rats died as soon as they started to lose weight. In the other five rats, the weight-loss curve was less characteristic.

Hematocrit. In control rats, hematocrit did not change over time, with a mean value of 50.31 ± 0.81%. The infected rats did not differ from controls at D_4 (control, 49.68 ± 2.03 vs infected rats, 49.29 ± 3.37%). However, a dramatic drop marked the second hematocrit measurement performed on D_9 (control, 51.05 ± 2.27 vs infected rats, 40.39 ± 5.04%; $P < 0.05$; Fig. 5). This value remained unchanged until the days preceding death. It is obvious that death did not occur systematically on the day of the hematocrit assessment. However, the last hematocrit measure may have been performed on the day of death (DD_0) or the day before (DD_{-1}) or even 2 or more days before. It appears that the hematocrit dropped significantly just before death occurrence with a decrease of −24.86 ± 8.04% at DD_0 and DD_{-1} vs −15.67 ± 7.41% at DD_{-2} or DD_{-3} when compared with D_4 ($P < 0.05$).

Open-Field Test. No difference was observed between the rats tested during the activity or the resting phases in both the control and infected groups. The Open-field test data were therefore pooled within each group.

Treatment (control [$n = 6$] vs infected [$n = 14$]) and time effects (days of test application) were observed for the total distance covered by the animals [57.01 ± 7.12 vs 50.68 ± 14.81, 48.67 ± 8.03 vs 38.24 ± 13.25, 42.33 ± 7.04 vs 28.36 ± 10.51 m for control and infected rats at D_3, D_8, and D_{15}, respectively; F value 5.26, $P = 0.03$ for the difference

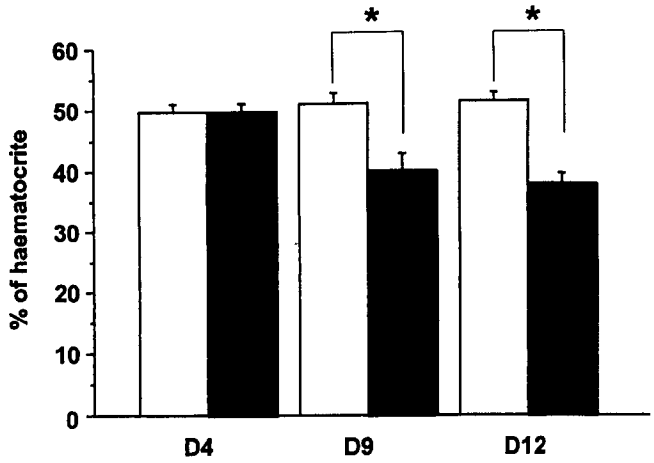

Figure 5. Percent hematocrit of control (white bars) and infected (dark bars) rats during the first (D4 post-infection), second (D9 post-infection), and third (D12 post-infection) measures. Data represent mean levels ± SD. Significant between-group differences. *Infected group versus respective control group ($P < 0.05$).

Figure 3. (A) Body weight gains in control and infected rats. Body weight is expressed as the pre-infection value at D0. Data points represent the mean ± SD. *Infected group versus respective control group ($P < 0.05$). (B) Body weight gains and losses in three infected rats with body-weight breakdown at different day before death. Grey diamond, rat that represents the most common profile; △, rat with one day of weight decrease; black diamond, rat with one daily weight gain in the decrease evolution of weight. Body weight is expressed as the pre-infection value at D0. Data points represent the mean ± SD. The control group is the same as in Figure 2A and is included here for comparison.

between both groups, and F value 18.46, $P < 0.001$ for the time effect (Fig. 6)]. However, the difference between both groups was significant only at D_{15} ($P < 0.05$; Fig. 6). For the "time spent in the corner" variable, a time effect (95% CI, d.f. 2, F value 3.26, $P = 0.05$) was observed without any treatment effect (F value 0.1; $P = 0.75$) or interaction (F value 1.22; $P = 0.31$) at the same time on D_3, D_8, and D_{15}.

However, performing the ANOVA test for the same dependent variables at the same time and D_{22} in animals surviving a time long enough ($n = 6$ for control and $n = 8$ for infected rats), a significant interaction between treatment and time was observed ($P < 0.05$).

The time before leaving the center of the Open-field after the animal was placed upon it (beginning of the exploratory behavior) did not change over time in the control group (1.83 ± 0.98 vs 1.51 ± 0.55 sec, at D_3 and D_{15}, respectively, $n = 6$) but increased during the last days of the disease in infected rats (2.38 ± 1.30 vs 3.75 ± 1.04 sec at D_3 and D_{15}, respectively, $n = 14$) leading to a significant difference between both groups (F value 15.09; $P = 0.002$).

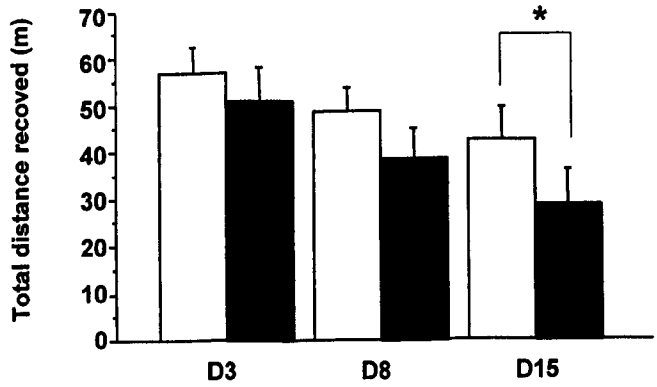

Figure 6. Mean locomotion (total distance covered during the 10-min session in m) in control (white bars) and infected rats (dark bars) during the first (D3 post-infection), second (D8 post-infection), and third (D15 post-infection) Open-field tests. Data represent mean levels ± SD. Significant between-group differences. *Infected group versus respective control group ($P < 0.05$).

Figure 4. Food consumption in control and infected rats. Data points represent the mean ± SD. *Infected group versus respective control group ($P < 0.05$).

This increase was even exacerbated at D_{22} (F value 14.71; $P = 0.004$, control $n = 6$, infected $n = 8$).

Histology. Trypanosomes and mononuclear inflammatory cells were clearly observed in the choroid plexus for the six infected rats studied at both D_{13} and D_{15} (Fig. 7); however, none of these elements were observed in the brain parenchyma.

Discussion

This study presents an experimental model of African trypanosomiasis in rats infected by *Trypanosoma brucei brucei*. To our knowledge, it is the first model to describe physiological and behavioral disturbances occurring in the course of the disease.

Drugs that are active during the hemolymphatic phase are inactive on the meningo-encephalitic phase because they do not cross the blood–brain barrier. Drugs that are active during the meningo-encephalitic phase are highly toxic. This is the reason why different animal models of trypanosome infection aimed at studying the therapeutic action of drugs have been proposed. It is therefore essential to be able to determine the time of the passage of trypanosomes into the CNS. However, until now, the gold standard to assess the stage of the disease has been the neuropathological examination (4).

We chose laboratory rodents because they allow easy handling and stabulation and present an acute infection that has strong similarities with the human disease. Isolates of *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodesiense* were used to produce infections with subacute or chronic course in mice and rats (15, 16). Such a model was developed for studying new drug therapies of meningo-encephalitic sequels of HAT (10, 13, 17, 18).

In our study, we used the *T. b. brucei* AnTat 1.1E strain, because it allows a long disease course (30 days on the average) with CNS involvement by around 21 days in mice (19). In rats, this strain is known to induce neuropathological abnormalities assessed by histology and electromicroscopic studies (20, 21).

Three major results shall be discussed: the acute weight loss observed at the end of the second week of infection related to a dramatic decrease in food consumption, the hematocrit profile, and the behavioral changes during the Open-field test.

Infected rats, contrary to control animals, experienced weight loss after a little less than 2 weeks of infection. A dramatic food intake drop, which was significant from the twelfth day of infection, always preceded the drop in body weight.

The modifications observed here in body weight and food resemble that of *T. b. gambiense*-infected patients during the meningo-encephalitic phase of HAT (7, 8), the patients dying in severe cachexia (2). Food intake disturbances are also observed in humans. Even though some rare cases of bulimia are observed, most often the patients are anorectic (7, 8). The loss of weight among domestic animals infected by trypanosomes is well known. It was also reported in rats infected by the *T. b. brucei* AnTat 1.1E subtype (22) as well as in rats infected by *T. b. gambiense* (23) and in rabbits infected with *T. b. brucei* (24). However, the briskness of the changes in body weight and food intake has not yet been described. It is therefore tempting to hypothesize that the entry of the trypanosome into the CNS is at the origin of these phenomena. The point of inflection of the food-intake curve could well signal the beginning of the meningo-encephalitic phase. Indeed, a recent study reported a decrease of body weight in infected rats during the meningo-encephalitic phase (20).

A significant decrease in hematocrit was measured early after the infection in all infected rats. It never occurred in control rats. In the majority of infected rats, this decrease was later followed by a sharp drop preceding death by a few days. Anemia has already been reported in rats infected by *T. b. brucei* (25) or *T. b. gambiense* (23). However, there is no report on the time course of this symptom in infected rats. Humans also develop a light anemia during the hemolymphatic phase of HAT (7, 8) and experience a dramatic drop in hematocrit at the terminal phase of HAT.

The initial decrease in hematocrit was observed during the first week after infection. It represents therefore the first sign of infection and may be linked to the immunoinflammatory process induced by the propagation of *T. b. brucei* during the hemolymphatic phase. It therefore could be considered as an index of this stage of the disease. The drop in hematocrit observed before death occurred probably

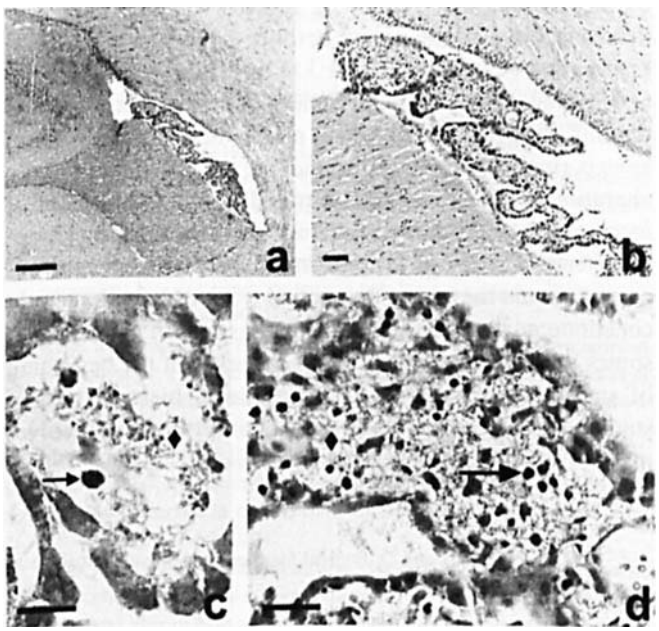

Figure 7. Lateral ventricle in a brain of a 13-day *Trypanosoma brucei brucei* AnTat 1.1E infected rat (a, bar = 500 μm, and b, bar = 100 μm). Presence of numerous trypanosomes [dark diamond] and few inflammatory cells (arrows) in the interstitial spaces of the choroid plexus in the same ventricle (c, bar = 25 μm, and d, bar = 50 μm). Hematein-eosin.

several days after the meningo-encephalitic phase had started in most animals.

Neurological alterations are represented by the altered parameters observed during the Open-field test. The total distance covered (motor activity) and latency to leave the central zone (exploratory behavior ability) became significantly different between both rat groups at D_{15} (3^{rd} test) and thereafter. The infected rats were also unable to maintain their exploratory behavior and remained prostrated in one of the corners of the apparatus, which parallels the lethargic behavior of patients at the meningo-encephalitic stage. We may therefore assume that the Open-field measures represent an index of the functional alterations induced during the meningo-encephalitic stage. Furthermore, the altered Open-field behavior appeared at the end of the second week of infection, when the weight loss consecutive to the decrease of food intake was observed.

In rodents, trypanosomes selectively enter brain areas with a reduced blood–brain barrier at relatively early stages of the infection (26). It has been proposed that a cornerstone in the pathology of *T. brucei* infection would be the injury to the choroid plexus (27). This has been noted in several studies with *T. b. gambiense* and *T. b. rhodesiense* in rodents and primates (28–30), and with *T. b. brucei* in mice (19). The parasites have therefore access to the cerebrospinal fluid (CSF) and subsequently, *via* the subarachnoidal spaces, to the perivascular extensions (Virchow-Robin spaces) into the brain. The parasite may then enter the CNS parenchyma. In our study, trypanosomes and some inflammatory cells were observed in the choroid plexus of rats from D_{13} post-infection. This agrees with our hypothesis that infected rats featuring abnormal Open-field behavior and reduced food-intake were likely to be in the meningo-encephalitic phase. The onset of CNS lesions observed in choroid plexuses of infected rats corresponds to a classical histological evolution of the disease in the CNS. This evolution is always continued by invasion of the brain parenchyma as it has been described in other chronic experimental models of HAT in mouse, rat, and monkey infected by *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodesiense* (1, 19, 30, 31–33).

Difference of parasite strains and their subsequent virulence may have non- negligible significances for the appearance of clinical and behavioral symptoms. However, the involvement of clinical criteria and concomitant presence of parasites in the plexus choroids may not be exclusive of our model with *T. b. brucei* AnTat 1.1E, but rather could be extrapolated to another experimental model using a different strain of *T. b. brucei*. In a study performed in rats with GVR 35/ C1.3, a low-virulence strain with the animals surviving more than 30 days, Philip *et al.* (34) observed a similar time course of disorders characterized by both a blood–brain barrier effraction and a weight loss. An accumulation of trypanosomes in the choroid stroma, with marked fragility and apparent damage to the choroid epithelium, was observed on day 21 p.i. Infected animals lost weight from day 28 post-infection (p.i.). Extensive damage of BBB with bilateral opening was observed consistently only in the late stages of the disease (day 35 and 40 p.i.) with parasites occasionally present in the parenchyma. Furthermore, rats infected with two different strains of *T. b. brucei*, GVR 35/ c.1 and EATRO 1216, featured a reduction in food intake from day 21 p.i. and for some individuals a weight decrease thereafter (35).

In *T. b. gambiense*-infected patients at the meningo-encephalitic phase, blood Tumor Necrosis Factor-α (TNF-α) concentrations are increased (36), this increase being concomitant to changes in neuroendocrine circadian rhythms (37–39) and related to the disease severity (40). The body-weight loss of trypanosome-infected rats with *T. b. brucei* AnTat 1.1E correlates with mRNA expression of pro-inflammatory cytokines interleukine-1-β (IL1-β) and TNF-α and with the progression of terminal and axonal degeneration in the brain (21). Thus, TNF-α may have caused the pronounced weight loss we observed.

Although TNF-α has a beneficial effect in HAT (41, 42), severe anemia and cachexia result from excessive TNF-α production (43) and are major causes of morbidity at the late stage of trypanosomiasis. It is now clear that IL1-β, interferon-γ (IFN-γ), and TNF-α decrease food intake by a direct effect on glucose-sensitive neurones of the hypothalamus (44) or indirectly through a stimulation of the hypothalamic prostaglandin E2 synthesis (45), which in turn stimulates the release of hypothalamic corticotropin-releasing factor, an endogenous anorexigenic neuropeptide (46).

The locomotor activity decrease may also be explained by the dramatic late release of TNF-α, which is known to decrease both spontaneous activity (47, 48) and social exploration (49).

In conclusion, the main finding of the present work was that infection with *T. b. brucei* affects brain functioning in an experimental rat model, mimicking the CNS- functioning alterations of HAT, as demonstrated by the impairment of locomotor activity and exploratory capacities during Open-field tests. The drops in food intake and body weight observed around the end of the second week of infection could constitute an accurate index of the passage of the trypanosomes in the CNS, therefore also a marker of the beginning of meningo-encephalitis. Nevertheless, histopathologic studies are needed to verify the possibility of the involvement of hypothalamic regulatory areas.

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