Persistent infection of Mongolian jirds with a non-pathogenic trypanosome, *Trypanosoma* (*Herpetosoma*) *grosi*

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(*Received 8 August 2002; revised 14 March 2003; accepted 27 April 2003*)

SUMMARY

Non-pathogenic trypanosomes of the subgenus *Herpetosoma* are normally host specific, and laboratory models include *Trypanosoma lewisi* in rats and *Trypanosoma musculi* in mice. Two isolates of *Trypanosoma grosi*, originating from *Apodemus agrarius* and *Apodemus peninsulae*, grew well in Mongolian jirds, *Meriones unguiculatus*, after intraperitoneal inoculation of 2×10^5 or a minimum 500 bloodstream forms. The course of *T. grosi* infection in jirds resembled *T. musculi* infection in mice, rather than *T. lewisi* infection in rats. At week 2 to 3 p.i. trypanosomes disappeared from the bloodstream, and neither prednisolone treatment nor splenectomy prevented parasite elimination from the bloodstream. However, these treatments induced a marked increase in peak parasite counts. Regardless of prednisolone treatment or splenectomy, all jirds after day 21 p.i. became resistant to the reinfection. Although no trypanosomes were detected in the bloodstream of recovered jirds, dividing parasites persisted in the medullary capillaries of the kidney, like *T. musculi* infection in mice. We propose the *T. grosi* infection in jirds as an additional laboratory model for the study of non-pathogenic trypanosomes.

Key words: *Trypanosoma grosi*, *Herpetosoma*, *Meriones unguiculatus*, laboratory model, life-cycle.

INTRODUCTION

Stercorarian trypanosomes of the subgenus *Herpetosoma* are host specific for their vertebrate hosts where they live extracellularly, primarily in the bloodstream (Hoare, 1972; Santos-Gomes *et al*. 1993; Noyes *et al*. 2002). Among these stenoxenous trypanosomes, *Trypansoma lewisi* and *Trypanosoma musculi* have been well studied in the laboratory, because they naturally parasitize rats of the genus *Rattus* and mice of the genus *Mus*, respectively. Both *T. lewisi* and *T. musculi* produce self-limiting asymptomatic infections and elicit life-long host immunity (Hoare, 1972; Mansfield, 1977; Viens, 1985; Teixeira, 1987). Infections caused by these 2 species evoke 2 serial immunological responses; the first response results in the arrest of parasite reproduction or eliminates the dividing forms from the peripheral blood (so-called 'first crisis'), while the second response eliminates the parasite from the circulation (so-called 'second crisis'). The mechanisms responsible for both responses have been studied intensively, and shown to differ between *T. lewisi* and *T. musculi* infections (Viens, 1985; Teixeira, 1987; Albright & Albright, 1991).

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Trypanosoma (*Herpetosoma*) *grosi* is a non-pathogenic trypanosome species parasitizing field and wood mice, *Apodemus agrarius* and *Apodemus sylvaticus* (Hoare, 1972). Information on the nature of *T. grosi* infection is limited due to limited availability of the natural hosts bred in the laboratory (Krampitz, 1959; Maraghi & Molyneux, 1989; Maraghi, Wallbanks & Molyneux, 1995). Recently, we succeeded in maintaining this protozoan species in the laboratory using Mongolian jirds, *Meriones unguiculatus*, without special manipulation of the parasite or the host. The course of infection with *T. grosi* in this laboratory host resembled *T. musculi* infection in mice, rather than *T. lewisi* infection in rats.

MATERIALS AND METHODS

Animals and parasites

Mongolian jirds were bred in the Institute for Animal Experiments, Hirosaki University School of Medicine. Six-week-old female SCID mice of CB-17 or BALB/c backgrounds were bred in the Central Institute for Experimental Animals, Japan. Female Hartley-strain guinea-pigs, 13 months old, were purchased from a commercial breeder (Funabashi Farm, Shizuoka, Japan). Fischer rats, 4 months old, were bred and provided by Professor T. Sato, Medical Technology College, Hirosaki University. All animal experiments were performed according

Parasitology (2003), **127**, 357–363. © 2003 Cambridge University Press
DOI: 10.1017/S0031182003003809 Printed in the United Kingdom

https://doi.org/10.1017/S0031182003003809 Published online by Cambridge University Press

to the Guidelines on Animal Experimentation as set out by Hirosaki University.

T. grosi were obtained as contaminants in primary cell cultures of tails from *A. agrarius* and *A. peninsulae* caught in Vladivostok, Russia, and maintained *in vitro* using RPMI 1640 medium (Nissui Pharmaceutical Co., Sugamo, Tokyo, Japan) supplemented with 0·3% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml amphotericin B, and 10% heat-inactivated foetal bovine serum (Filtron, Export Dve, Brooklyn, Australia) under sterile conditions of 5% CO_2 at 37 °C. The original cultures containing trypanosomes were kindly provided by Professor Y. Obara, Faculty of Agriculture and Life Science, Hirosaki University, and referred as SESUJI and HANTO isolates, that originated in tissue cultures from *A. agrarius* (Sesuji-nezumi in Japanese) and *A. peninsulae* (similarly, Hanto-nezumi), respectively. After confirmation that *T. grosi* can proliferate in jirds, the parasite was maintained by weekly intraperitoneal injection of $2{\cdot}0 \times 10^5$ bloodstream-form trypanosomes into jirds that were also injected s.c. with 10 mg of prednisolone tertiary-butylacetate (Suspension of Codelcortone® – T.B.A., Merck & Co., Inc., Rahway, New Jersey, USA) at infection.

Immunosuppressive treatment

To evaluate the effects of immunosuppressive treatments on the growth of *T. grosi*, jirds were splenectomized and/or treated with prednisolone. Splenectomy was carried out 1 week before infection. Jirds were treated with 10 mg of prednisolone on the day of infection as described above. In addition to a single prednisolone treatment, a group of jirds was repeatedly treated with 10 mg of prednisolone on days 10, 7, 4 and 0 prior to infection.

Infection and monitoring of parasitaemia

Parasite preparations for infection of jirds were isolated from prednisolone-treated jirds on day 7 p.i. and resuspended in heparin-containing supplemented RPMI 1640 medium without foetal bovine serum at a concentration of $1{\cdot}0 \times 10^6$ organisms/ml. Animals were injected i.p. with 0·2 ml of this suspension ($2{\cdot}0 \times 10^5$ organisms/animal), unless otherwise stated. Those for infection of mice, rats and guinea-pigs were isolated from *in vitro* cultures to avoid the graft *vs* host reaction due to contaminated leukocytes in parasite preparations from jird blood. We have never experienced noticeable differences in infectivity and the course of infection between parasite preparations from jird bloods and *in vitro* cultures when infection was made to jirds. Two groups of 5 SCID mice each of different genetic background were inoculated i.p. with $5{\cdot}0 \times 10^6$ trypanosomes. A group of 3 rats was inoculated i.p. with $1{\cdot}0 \times 10^6$ trypanosomes, and a group of 2 guinea-pigs was injected i.p. with $1{\cdot}5 \times 10^5$. These experiments were carried out for both isolates. Details of the experiments using jirds were mentioned below in the Results section.

The course of parasitaemia was monitored by counting the number of trypanosomes in blood on a haemocytometer. The blood sample was collected from the orbital venous plexus and diluted in 0·83% NH_4Cl–Tris buffer with heparin. Morphological examination and measurements of the dimensions of the 2 isolates were conducted using a thin blood smear obtained from prednisolone-treated jirds after staining with Giemsa's solution, with the aid of *camera lucida*. To determine the presence of dividing parasites in the kidney, impression smears were obtained and stained with Giemsa's solution.

In vitro *culture*

In vitro cultures of bloodstream forms of *Herpetosoma* trypanosomes are successfully maintained for long periods by using mammalian feeder cells or a conditioned medium from murine macrophages grown in the presence of 2-mercaptoethanol (Albright & Albright, 1980; Vincendeau *et al.* 1986; Mohamed *et al.* 1988; Behr, Mathews & D'Alesandro, 1990). In RPMI 1640 medium supplemented with heat-inactivated 10% foetal bovine serum, *T. grosi* grew well attaching to the primary culture of tail cells from *Apodemus* spp. by means of their flagella. Based on this property, we prepared the primary cell culture of kidneys taken from 2-week-old jirds in culture flasks (SUMILON 250 ml flask; Sumitomo Bakelite Co., Ltd, Shinagawa, Tokyo, Japan) for maintenance of the parasites. With use of the feeder cells, trypanosomes proliferated well, and a huge number of trypanosomes appeared freely in the medium. The culture without feeder cells resulted in no increase of parasites. Growth of trypanosomes was checked under an inverted microscope, and the medium was changed every 3–4 days. Parasites used for infection were prepared from trypanosomes free in the medium.

Antiserum to T. grosi

To obtain anti-trypanosome sera, guinea-pigs examined for their susceptibility to *T. grosi* were repeatedly immunized by reinfection with $1{\cdot}0 \times 10^5$ to $2{\cdot}0 \times 10^7$ of each isolate on days 15, 22 and 36 after the primary injection. Sera were collected 8 days after the last injection. Elevation of specific IgG titres was confirmed by an enzyme-linked immunosorbent assay (ELISA) using crude trypanosome antigens described below and peroxidase-conjugated goat serum to guinea-pig IgG (E-Y Laboratories, Inc., San Mateo, California, USA). The assay was carried out according to a standard procedure, as described previously (Sato *et al.* 1996).

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Parasite antigens

Cultured trypanosomes of SESUJI and HANTO isolates were washed several times in supplemented RPMI medium without foetal bovine serum, and immersed in 50 mM Tris buffer, pH 8·2, containing 1% Triton X-100, 150 mM NaCl, 10 mM EDTA and 0·5 mM phenylmethylsulfonyl fluoride. After 3 cycles of freezing and thawing, the samples were subjected to intermittent ultrasonication for 5 min. Supernatant was collected by centrifugation at 6000 ***g*** for 30 min, and the protein concentration was determined using Bio-Rad protein assay solution (Bio-Rad Lab., Richmond, CA, USA).

Immunohistochemistry

Cryostat sections, 6 μm thick, of the kidney, liver and spleen were air-dried and fixed for 10 min in cold acetone. To reduce the activity of endogenous peroxidase, sections were immersed in acetone containing 0·02% H_2O_2. To block non-specific binding sites, sections were incubated for 1 h with phosphate-buffered saline (PBS) containing 10% normal goat serum. Anti-serum to trypanosomes and the conjugate were diluted in PBS containing 0·05% (v/v) Tween 20® and 10% normal goat serum. Each reaction was continued for 1 h at room temperature. Bound antibody was detected using colour development by 3,3′-diaminobenzidine, followed by light counterstaining with haematoxylin. In order to assess non-specific staining, control sections were prepared as above but with serum from naive guinea-pigs.

Statistical analysis

Differences between 2 groups were examined for significance using the Student's *t*-test. A *P* value less than 0·05 denoted statistical significance.

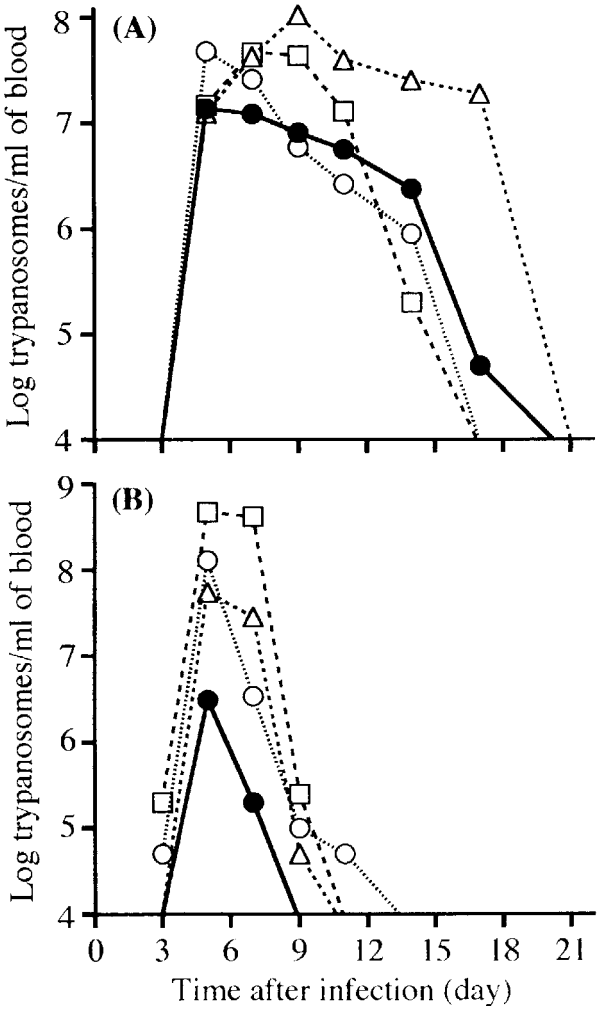

Fig. 1. Time-course of parasitaemia following intraperitoneal inoculation of 2×10^5 *Trypanosoma grosi* of SESEJI (A) and HANTO (B) isolates in 12-week-old female jirds with/without immunosuppressive treatments; naive (●), prednisolone-treated (○), splenectomized (△), and prednisolone-treated, splenectomized (□). Each value is the average of log trypanosomes/ml of blood from 2 animals/group.

RESULTS

Intraperitoneal inoculation of *T. grosi* caused parasitaemia exclusively in jirds, whereas SCID mice, rats and guinea-pigs were completely resistant to the infection with either isolate of trypanosomes. Therefore, further observations were conducted using only jirds.

Course of parasitaemia in jirds after intraperitoneal inoculation

Although jirds were susceptible to both SESUJI and HANTO isolates of *T. grosi*, the course of parasitaemia with these 2 isolates differed (Fig. 1). After a latent period of approximately 3 days, an exponential growth phase of the parasites was noted. Parasitaemia peaked on days 5–9 or days 5–7 after intraperitoneal inoculation of SESUJI and HANTO isolates, respectively. This was followed by a rapid clearance of the parasites from the blood to levels below those detectable by microscopy, which was completed by day 14 p.i. in jirds infected with HANTO isolate, in contrast to day 21 p.i. in jirds infected with SESUJI isolate (Fig. 1). Challenge inoculation of the homologous isolate to these jirds on day 21 p.i. produced no parasitaemia when blood samples were checked on days 3, 5 and 7 after the challenge infection.

Fig. 2 compares the effects of single treatment or serial pre-treatment of jirds with prednisolone on the level and duration of parasitaemia. Serial treatment with prednisolone from day 10 prior to infection did not affect significantly the latent period and the duration of parasitaemia, but it did increase the level of peak parasitaemia. Although these 2 isolates showed different courses of infection even under

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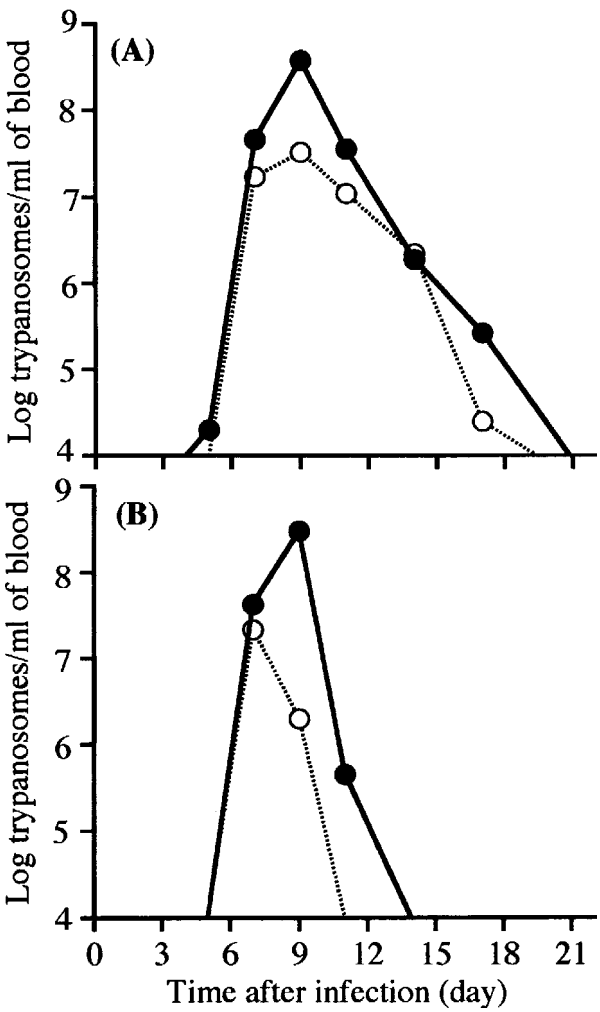

Fig. 2. Time-course of parasitaemia following intraperitoneal inoculation of 2×10^5 of *Trypanosoma grosi* of SESEJI (A) and HANTO (B) isolates in 12-week-old male jirds with single or serial prednisolone treatment. Jirds were treated singly with 10 mg of prednisolone at infection (○), or repeatedly with 10 mg of prednisolone from day 10 prior to the infection (●). Each value is the average of log trypanosomes/ml of blood of 5 animals/group.

conditions of immunosuppression, morphological examination and measurement of trypanosomes obtained from prednisolone-treated jirds on day 6 p.i. showed no difference between the 2 isolates (Table 1 and Fig. 3).

To examine for host-sex differences in the susceptibility to *T. grosi* infection, 14-week-old female and male jirds were inoculated i.p. with $2{\cdot}0 \times 10^5$ and $2{\cdot}0 \times 10^4$ parasites of SESUJI isolate (4 animals/group), and parasitaemia was checked on days 6, 10 and 14 p.i. Higher parasite counts were detected in female jirds than in males, but the difference was not statistically significant at both inoculation doses. To determine the minimum dose necessary to induce parasitaemia of *T. grosi*, 20-week-old male jirds were inoculated i.p. with $1{\cdot}0 \times 10^6$, $2{\cdot}0 \times 10^5$, $2{\cdot}0 \times 10^4$, $2{\cdot}0 \times 10^3$ and 500 parasites of HANTO isolate (3–4 animals/group), and parasitaemia was checked on days 6, 10, 14, 18 and 22 p.i. Parasitaemia was observed in all animals on day 6 p.i., but no parasites were detected on day 10 p.i. in blood samples of jirds inoculated with fewer than 2×10^5 parasites. No jirds showed parasitaemia on day 14 p.i. or later.

Persistence of proliferating parasites in the kidney

To check for the persistence of dividing parasites in the kidney, which is known to occur in *T. musculi* infection in mice (Viens *et al.* 1972; Wilson *et al.* 1973; Targett & Viens, 1975), two 12-week-old female or male jirds used in the laboratory maintenance of each isolate of *T. grosi* were sacrificed at weekly intervals from weeks 1 to 5 p.i. Cryostat sections of the kidney, liver and spleen, as well as impression smears of the kidney, were prepared for immunohistochemistry and Giemsa's staining. Aggregates of parasites were noted in the medullary capillaries of kidneys from all animals (Fig. 4), and rarely in the spleen. Occurrence of such packed parasites in the medullary capillaries of prednisolone-treated jirds became less frequent with time, and was infrequent in untreated jirds when compared with immunosuppressed jirds (data not shown). No parasites were detected in the liver. The impression smears of the kidney contained dividing parasites showing variable morphology (Fig. 5).

DISCUSSION

T. lewisi, which naturally infects rats, can also infect other rodents such as mice, Mongolian jirds and guinea-pigs, when large inocula of dividing bloodstream forms are used, coupled with daily administration of rat serum (Mühlpfordt, 1968, 1969). However, the duration of parasitaemia in such infections is shorter than in the natural host. Furthermore, even after serial passages in mice for 16 months, *T. lewisi* did not adapt to or survive in mice without daily administration of rat serum (Mühlpfordt, 1968). In contrast to the above findings with *T. lewisi*, our results demonstrate that *T. grosi* can infect Mongolian jirds without special manipulation of the parasite or the host.

Mühlpfordt (1969) succeeded in establishing parasitaemia in jirds using 18 serial passages of *T. lewisi* for 67 days, with inoculum doses of mostly $1{\cdot}6$–$9{\cdot}3 \times 10^6$. The mean duration of the patent period was 5·3 days (range 3–7 days). Parasitaemia in these animals was characterized by polymorphism of the parasite, lack of a chronic stage in which a uniform size of trypanosomes was seen, and abrupt disappearance of the parasite from the bloodstream. In contrast, we have demonstrated that *T. grosi* can produce parasitaemia in jirds using an inoculum dose of $2{\cdot}0 \times 10^5$, or a minimum 500, and a chronic stage is established, which closely resembles the infection

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Table 1. Morphological features of different isolates of *Trypanosoma grosi* (measurements in μm)

	SESUJI isolate from *Apodemus agrarius*	HANTO isolate from *Apodemus peninsulae*
PN *vs* NA	PN > NA	PN > NA
NI	1·31 ± 0·44	1·11 ± 0·18
PK *vs* KN	PK < KN	PK < KN
KI	1·38 ± 0·10	1·38 ± 0·08
K	0·98 × 0·70	0·87 × 0·56
F	8·63 ± 1·44	8·34 ± 0·93
L	30·18 ± 3·14	31·55 ± 1·65
W	2·10 ± 0·35	2·16 ± 0·44

(Abbreviations: PN, the distance from the posterior end (P) to midnucleus (N); NA, the distance from midnucleus to the anterior end (A); NI, the nuclear index (NI = PN/NA); PK, the distance from the posterior end to the kinetoplast (K); KN, the distance from kinetoplast to midnucleus; KI, the kinetoplast index (KI = PN/KN); L, the total length including the free flagellum (F); W, maximum width.)

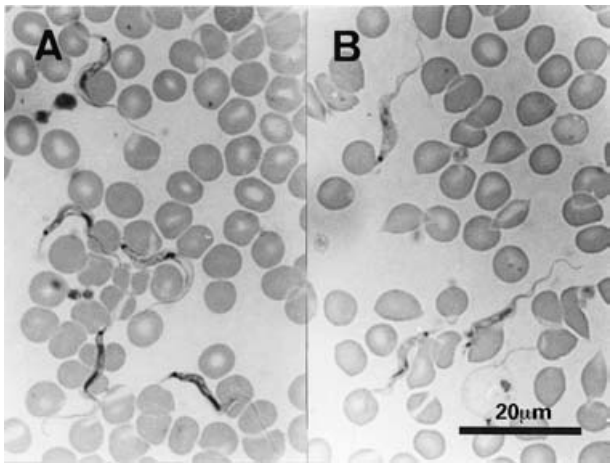

Fig. 3. *Trypanosoma grosi* in the peripheral blood of prednisolone-treated jirds on day 6 p.i. (A) SESUJI isolate; and (B) HANTO isolate. Giemsa's stain.

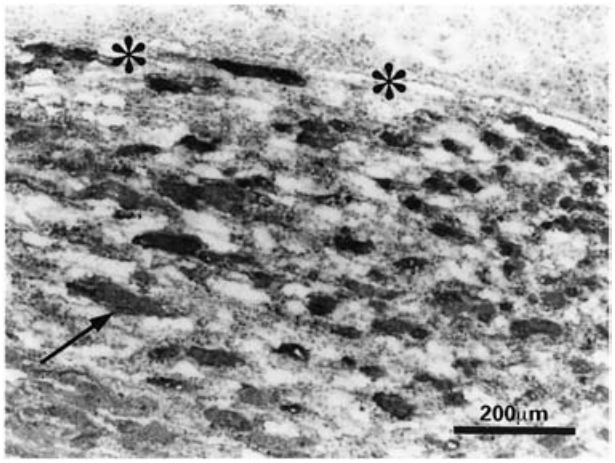

Fig. 4. Persistent *Trypanosoma grosi* (SESUJI isolate) in the medullary capillaries of the kidney of a prednisolone-treated jird at week 5 p.i., as demonstrated by immunohistochemistry. Asterisks indicate the margin of pyramis renales, and multiple dark patches scattering in the medulla are aggregations of dividing forms (an arrow indicates one example).

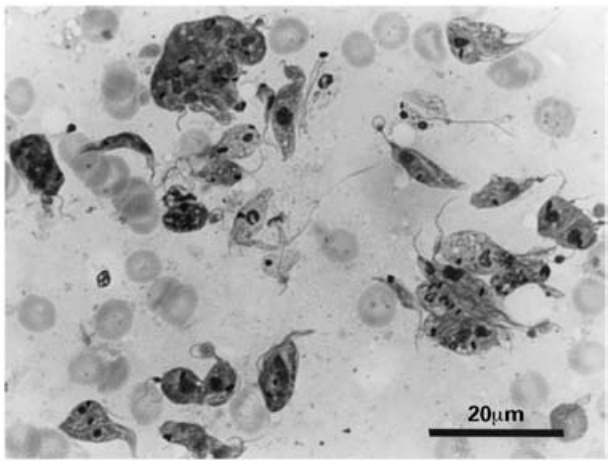

Fig. 5. Impression smear of dividing forms of *Trypanosoma grosi* (SESUJI isolate) in the medullary capillaries of the kidney from a prednisolone-treated jird at week 5 p.i. Giemsa's stain.

course of *T. musculi* in the natural host. Our results also indicate that the duration of parasitaemia of *T. grosi* in jirds is not shorter than that reported in the natural host, *A. sylvaticus* (Maraghi & Molyneux, 1989). Although different courses of parasitaemia were noted in jirds inoculated with SESUJI and HANTO isolates of *T. grosi*, these two had identical morphology and dimensions, coincident with the previous description of *T. grosi* in the natural host (Krampitz, 1961). The same kind of a difference in the course of parasitaemia has been reported in different isolates of *T. musculi* (Chiejina *et al.* 1993). Alternatively, since Noyes *et al.* (2002) recently detected *Herpetosoma* trypanosomes of a novel genotype distinct from *T. grosi* from *A. sylvaticus*, we should consider such a possibility for our 2 isolates until genetic confirmations have done.

To examine the effects of immunosuppression on the course of *T. grosi* infection in jirds, we investigated the effects of treatment with prednisolone

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and/or splenectomy. Prednisolone treatment and/or splenectomy resulted in higher levels of peak parasitaemia, but did not prolong remarkably the duration of patent parasitaemia. Treated animals still showed resistance to the homologous reinfection in a manner similar to that observed in untreated animals. It has been reported that both cortisone treatment and splenectomy of rats prolong the reproductive phase and increase the level of parasitaemia of *T. lewisi* (Sherman & Ruble, 1967; Greenblatt, Spira & Tyroler, 1972; Dusanic, 1975).

Dividing forms of *T. musculi* have been detected in the kidney of immune mice almost 1 year after recovery from patent parasitaemia (Viens *et al*. 1972; Wilson *et al*. 1973; Targett & Viens, 1975). Similar forms have been found in the kidney of rats infected with *T. lewisi*, but only during the patent reproductive phase in the blood and not during the chronic phase or after recovery from parasitaemia (Wilson *et al*. 1973; Targett & Viens, 1975). Drug-induced elimination of *T. musculi* remaining in the vasa recta of the kidney enabled reinfection, indicating that the long-term survival of *T. musculi* in the kidney of the immune host plays an important role in maintaining immunity against reinfection (Oliver & Viens, 1985). When the recovered mice become pregnant, the parasites leave their refuge in the kidney, causing patent parasitaemia (Viens, Roger & Dubois, 1983). Persistent dividing forms of *T. grosi* in jirds that recovered from parasitaemia suggests that the immune mechanism(s) acting against *T. grosi* infection probably resemble those against *T. musculi* infection in mice rather than those against *T. lewisi* in rats.

In both *T. musculi* and *T. lewisi* infections in the natural hosts, humoral immune responses play a major role in inhibiting parasite reproduction and their elimination from the bloodstream (Giannini & D'Alesandro, 1982; D'Alesandro, 1975, 1986; Desbiens & Viens, 1981; Vargas *et al*. 1984; Wechsler & Kongshavn, 1985, 1986; Viens, 1985; Teixeira, 1987; Albright & Albright, 1991). Thymus-dependence of the response is noted only in infections caused by *T. musculi* (Viens *et al*. 1974; Hanson & Chapman, 1974; Targett & Viens, 1975; House & Dean, 1988*a*, *b*). The role played by T-cells in *T. grosi* infection in jirds seems, however, to be different from *T. musculi* infection in mice; parasitaemia of *T. musculi* continued in T-cell-depleted mice, whereas T-cell depletion could not prevent the recovery of infected jirds from parasitaemia with *T. grosi* (Sato, Ito & Kamiya, unpublished observations).

Intriguing points to be pursued still remain in rodent trypanosomes of the subgenus *Herpetosoma* as has been mentioned previously by Albright & Albright (1991), but all published information is based on the only 2 laboratory models so far described, *T. lewisi* in rats and *T. musculi* in mice. We propose the *T. grosi* infection in jirds as an additional laboratory model for the study of *Herpetosoma* trypanosomes.

This work was supported in part by Grants-in-Aid (Nos. 13575039 and 13670240) from the Japan Society for the Promotion of Science. We would like to acknowledge the Japan Health Science Foundation for a scholarship to H. Sato, that enabled valuable discussion with Professor John R. Kusel, IBLS, University of Glasgow on this work.

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https://doi.org/10.1017/S0031182003003809 Published online by Cambridge University Press