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Neurotropic behaviour of *Trichobilharzia regenti* in ducks and mice

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Abstract

The bird nasal schistosome *Trichobilharzia regenti* is a new agent of cercarial dermatitis. Cercariae are able to penetrate the skin of birds and mammals including man. The parasite then attacks the central nervous system. The present study has shown that schistosomula avoid penetration of blood capillaries and enter the peripheral nerves of the legs of mice and ducks as early as 1 day post-infection (p.i.) and 1.5 days p.i., respectively. These peripheral nerves are used as a route to the spinal cord. In the specific host (duck) schistosomula were found in the spinal cord from 2 days p.i. until 15 days p.i. and in the brain from 12 days p.i. until 18 days p.i. In non-specific hosts (mice; inbred strains BALB/c, hr/hr, SCID) living schistosomula were found in the spinal cord from 2 days p.i. until 21 or 24 days p.i. (depending on the mouse strain) and in the brain of two (BALB/c, SCID) of three inbred strains from 3 days p.i. until 24 days p.i. No correlation was found between the infection dose and clinical status of the experimental hosts. A high affinity of schistosomula for the peripheral nerves was also proved *in vitro*, suggesting a new type of migratory behaviour in schistosomatids.

Introduction

Parasitic worms can be divided into two main groups: cavity- and tissue-dwelling helminths, the latter occupying different parts of the body. Some helminths are adapted to the life in the central nervous system (CNS; brain and spinal cord) and in this location, they can be found as facultative (e.g. larvae of *Taenia solium*, *Echinococcus* spp., *Toxocara canis*) or obligate (e.g. *Diplostomum phoxini*, *D. baeri*, *Ornithodiplostomum ptychocheilus*) parasites. Invasion of the CNS represents a health risk to the host as neuromotor functions can substantially be impaired, with the host being susceptible to predators. In this way parasite transmission to the next host can be facilitated.

In the family Schistosomatidae it is commonly accepted that cercariae penetrate the skin, transform into schistosomula and enter the blood capillaries of their hosts. Schistosomula and adult worms then spend their life in the blood vessels and they are considered to be true blood parasites. Nevertheless, recent data show that some species (*Trichobilharzia regenti*) parasitizing birds can be found extravassally and schistosomula migrate through the spinal cord and brain to the nasal cavity where sexual maturation, mating and egg laying occur (Horák *et al.*, 1999). Moreover, *T. regenti* has the ability to infect mammals and also migrates to the CNS although neither maturation nor egg laying takes place (Horák & Kolářová, 2001). This therefore raises two questions relating to the migratory behaviour of *T. regenti*: (i) how does the parasite find and enter the CNS? and (ii) are there any differences in the parasite CNS migration in various animal hosts? One can hypothesize that bird schistosomes such as *T. regenti* enter capillaries (as in the case of human schistosomes) and migrate via the circulatory system to the lungs and then reach the spinal cord via the blood supply (Horák *et al.*, 1999), or nasal bird schistosomes may use other routes in order to reach the spinal cord and these need to be investigated. The present study, therefore, which expands on the work of Horák *et al.* (1999) aims to produce a precise description of *T. regenti* migration in ducklings and three mice strains, including one immunocompromised strain.

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Materials and methods

The experimental hosts were 5- to 10-day-old ducklings of *Anas platyrhynchos* f. dom., two immunocompetent inbred mouse strains (3-month-old BALB/c and 3- and 5-week-old hr/hr) and a 1-month-old immunocompromised inbred SCID strain. The legs of ducklings and the tails and legs of BALB/c mice were immersed for 1 h in water containing cercariae of *T. regenti* and tails and legs of hr/hr and SCID mice were immersed for 15 or 30 min. Exposure doses were 1500–8000 cercariae for ducklings, 1500–10000 cercariae for BALB/c mice, 150–800 cercariae for hr/hr and SCID mice exposed for 15 min and 3000–7000 cercariae for hr/hr and SCID mice exposed for 30 min. The number of penetrating worms was expressed as the number of shed tails. The migration of schistosomula was evaluated at 1, 2, 3, 6, 9, 12, 15 and 18 days post-infection (p.i.) in ducklings, 1, 2, 3, 6, 9, 12, 15, 18, 21 and 24 days p.i. in BALB/c mice and 1, 2, 3, 9, 15, 21 and 24 days p.i. in hr/hr and SCID mice. Three ducklings and three BALB/c mice were examined at the times mentioned above. Two SCID and two hr/hr mice were examined on days 1, 2 and 3 p.i. and one SCID and hr/hr mouse was examined in the later phases of the infection (9, 15, 21, 24 days p.i.). Because of heavy infection symptoms and early death in some cases, a total of 24 ducklings, 32 BALB/c, 10 hr/hr and 10 SCID mice remained for examination. Squashed preparations of the skin (tail of mice, legs of ducks and mice), peripheral nerves of the legs, spinal cord, brain, lungs, blood, liver, kidney and spleen were examined under a light microscope and the number of worms assessed.

A high affinity of cercariae/schistosomula of *T. regenti* for the nervous system was also tested *in vitro*. Twice concentrated Tris buffer (40 mM Tris, 300 mM NaCl, pH 7.8) was mixed with the same volume of distilled water containing freshly emerged cercariae and cercariae were incubated in this solution for 5 h at 37°C in order to transform to schistosomula. The peripheral nerves (*nervus metatarsus dorsalis*, *n. metatarsus intermedius*, *n. metatarsus lateralis* and *nervi digitales laterales*) of duckling legs were freshly dissected out, placed on a slide and incubated with 15–25 worms in Tris buffer in a wet chamber at 37°C for 45 min. A squashed tissue preparation was then examined under the light microscope. The attraction of schistosomula to various blood vessels was tested in a similar way: the *vena metatarsea anterior lateralis*, *v. metatarsea anterior medialis*, *v. metatarsea anterior anterior* and *venae digitorum laterales* were dissected out from the legs of ducklings, immersed in Tris buffer containing schistosomula at 37°C for 45 min and examined under the light microscope. These experiments were performed three times.

Results

The duck model (specific host)

Generally, the number of penetrating cercariae was 800–5000 per host, with a penetration success of 22–95%. In the case of five ducklings (20.8%), the onset of leg paralysis was recorded on day 7 p.i. Two of five hosts showed weak leg paralysis at an infection dose of 1000 cercariae per animal and in three ducklings a severe leg paralysis developed, where the number of penetrating cercariae was 1000 cercariae in one duckling and 3000 cercariae in each of two ducklings.

Schistosomula were found in the skin, peripheral nerves, spinal cord, medulla oblongata, hemispheres, bulbus olfactorius, nasal cavity and the lungs (table 1). One duckling was examined on 1.5 days p.i. and worms appeared in the skin and peripheral nerves of legs (in the *nervi digitales laterales et mediales*, *n. metatarsus dorsalis lateralis* and *medialis et intermedius*) (fig. 1a). Schistosomula remained in the peripheral nerves (*n. peronaeus profundus*, *superficialis et communis*, *n. tibialis*, *n. ischiadicus* and *n. femoralis*) until day 3 p.i. Worms were found in the synsacral and thoracic spinal cord from day 2 p.i. until day 15 p.i. and in the cervical spinal cord from day 6 p.i. until day 15 p.i. Worms occurred in the medulla oblongata on day 12 p.i. and migrating worms reached the hemispheres on day 15 p.i. Worms appeared in the

Table 1. The occurrence of *Trichobilharzia regenti* in ducklings up to 18 days post-infection (p.i.).

Days p.i.	1	2	3	6	9	12	15	18
Mean no. of penetrating worms	3940	2177	1875	1983	2910	1788	1698	2277
Mean no. of worms in tissues	112*	29	74	106	144	104	43	11
Skin	100*	0	0	0	0	0	0	0
Lungs	0	2	0	0	0	0	0	0
Peripheral nerves	12	19	5	0	0	0	0	0
Synsacral spinal cord	0	6	59	91	69	55	14	0
Thoracic spinal cord	0	2	10	14	46	23	16	0
Cervical spinal cord	0	0	0	1	29	25	9	0
Medulla oblongata	0	0	0	0	0	1	0	0
Cerebellum	0	0	0	0	0	0	0	0
Hemispheres	0	0	0	0	0	0	4	0
Bulbus olfactorius	0	0	0	0	0	0	0	1
Nasal cavity	0	0	0	0	0	0	0	10

The number of worms is the mean value (decimals values rounded off) from three infected ducklings.
*Estimated number.

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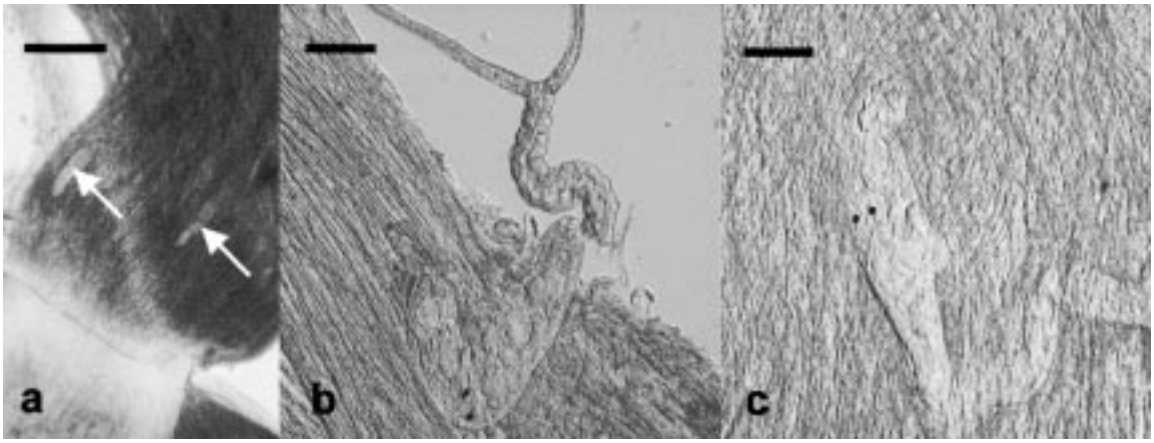

Fig. 1. Schistosomula of *Trichobilharzia regenti* in the peripheral nerves of a duckling leg. (a) In the *nervus peronaeus communis* 2 days p.i. (scale bar 300 μm). (b, c) Transformed schistosomula after penetration into the *nervus peronaeus communis* under *in vitro* conditions (scale bar 50 μm).

bulbus olfactorius and nasal cavity on day 18 p.i. Mature adults, eggs and miracidia were found in the nasal cavity only. Other organs and tissues were parasite free, except for the lungs where migrating schistosomula were found in two ducklings 2 days p.i.

Mouse models (non-specific host)

In the case of BALB/c mice, the number of penetrating cercariae was 300–2000 per host, with a penetration success of 19–88% and schistosomes were established in 28 of 32 mice examined. Leg paralysis was not observed. Schistosomula were found exclusively in the skin, peripheral nerves, spinal cord, medulla oblongata, cerebellum, hemispheres and lungs (table 2). In the peripheral nerves, worms were found at 2 days p.i. (in the *n. peroneus communis*, *n. saphena*, *n. cutaneus surae caudalis*, *n. tibialis*, *n. ischiadicus* and *n. femoralis*). Schistosomula were recorded in the lumbar spinal cord from day 2 p.i. until day 18 p.i., the thoracic spinal cord from day 2 p.i. until day 21 p.i. and the cervical spinal cord from day 3 p.i. until day 15 p.i. Living worms were found in the medulla oblongata from day 3 p.i. until day 15 p.i. and in the cerebellum on days 6, 15 and 18 p.i. In the hemispheres worms were present from day 12 p.i. until day 24 p.i. The bulbus olfactorius and the nasal cavity did not contain any developing worms. The lungs were also worm free except in one mouse at 3 days p.i. (table 2).

In the case of hr/hr mice, 80–400 cercariae (54–78%) and 600–2000 cercariae (39–88%) achieved penetration success after 15 min and 30 min exposure, respectively. Schistosomes became established in 9 of 10 mice. Leg paralysis was not observed. Schistosomula were found exclusively in the skin, peripheral nerves, spinal cord and medulla oblongata of mice (table 3). Worms appeared in the peripheral nerves (*n. ischiadicus* and *n. femoralis*) on days 2 and 3 p.i. and invaded the lumbar, thoracic and

Table 2. The occurrence of *Trichobilharzia regenti* in the inbred mouse strain BALB/c up to 24 days post-infection (p.i.).

Days p.i.	1	2	3	6	9	12	15	18	21	24*
Mean no. of penetrating worms	2756	2585	1932	1587	1276	1643	2347	2818	2461	2488
Mean no. of worms in tissues	90**	9	13	12	7	10	7	4	2	1
Skin	90**	0	0	0	0	0	0	0	0	0
Lungs	0	0	1	0	0	0	0	0	0	0
Peripheral nerves	0	6	0	0	0	0	0	0	0	0
Lumbar spinal cord	0	2	4	1	1	2	2	1	0	0
Thoracic spinal cord	0	1	6	5	3	3	2	1	1	0
Cervical spinal cord	0	0	1	3	2	1	1	0	0	0
Medulla oblongata	0	0	1	2	1	3	1	0	0	0
Cerebellum	0	0	0	1	0	0	1	1	0	0
Hemispheres	0	0	0	0	0	1	0	1	1	1
Bulbus olfactorius	0	0	0	0	0	0	0	0	0	0
Nasal cavity	0	0	0	0	0	0	0	0	0	0

The number of worms is the mean value (decimals values rounded off) from three infected mice.
*Only one mouse examined.
** Estimated number.

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Table 3. The occurrence of *Trichobilharzia regenti* in the inbred mouse strain hr/hr up to 24 days post-infection (p.i.).

Days p.i.	1	2	3	9*	15*	21*	24*
Mean no. of penetrating worms	640	418	520	358	265	252	190
Mean no. of worms in tissues	110**	18	38	17	7	0	2
Skin	110**	0	0	0	0	0	0
Lungs	0	0	0	0	0	0	0
Peripheral nerves	0	5	2	0	0	0	0
Lumbar spinal cord	0	6	9	3	1	0	0
Thoracic spinal cord	0	6	19	8	4	0	2
Cervical spinal cord	0	1	8	4	2	0	0
Medulla oblongata	0	0	1	2	0	0	0
Cerebellum	0	0	0	0	0	0	0
Hemispheres	0	0	0	0	0	0	0
Bulbus olfactorius	0	0	0	0	0	0	0
Nasal cavity	0	0	0	0	0	0	0

The number of worms is the mean value (decimals values rounded off) from two infected mice.
*Only one mouse examined.
**Estimated number.

cervical spinal cords from day 2 to day 15 p.i. In one mouse worms were found in the thoracic spinal cord on day 24 p.i. Schistosomula were also localized in the medulla oblongata from day 3 p.i. until day 9 p.i.

In the case of SCID mice, 80–400 cercariae (56–90%) and 600–2000 cercariae (20–89%) achieved penetration success after 15 min and 30 min exposure, respectively. Schistosomes became established in 8 of 10 mice. Leg paralysis was not observed. Schistosomula were found exclusively in the skin, peripheral nerves, spinal cord, medulla oblongata, hemispheres and bulbus olfactorius of mice (table 4). Schistosomula appeared in the peripheral nerves (*n. ischiadicus* and *n. femoralis*) on days 2 and 3 p.i. and in one mouse on day 1 p.i. (*n. ischiadicus*). The spinal cord was infected from day 2 p.i. until day 21 p.i. in the case of the lumbar and thoracic spinal cord and until day 15 p.i. in the cervical spinal cord. In the medulla oblongata schistosomula were localized from day 3 p.i. until day 21 p.i. and in the cerebellum from day 3 p.i. until day 15 p.i. The hemispheres contained worms from day 9 p.i. until day 21 p.i. and the bulbus olfactorius from day 15 p.i. until day 21 p.i.

In vitro *penetration of peripheral nerves*

The affinity of schistosomula for excised duckling peripheral nerves was tested three times. On two occasions schistosomula actively moved to the nerves, with about a third of schistosomula attaching to the nerves and about a fifth penetrating into the nerves (fig. 1b, c). The affinity of schistosomula for blood vessels was tested in the same way, but no interaction was observed.

Table 4. The occurrence of *Trichobilharzia regenti* in the inbred mouse strain SCID up to 24 days post-infection (p.i.).

Days p.i.	1	2	3	9*	15*	21*	24*
Mean no. of penetrating worms	2828	486	1014	285	320	260	81
Mean no. of worms in tissues	50**	12	19	22	32	7	0
Skin	50**	0	0	0	0	0	0
Lungs	0	0	0	0	0	0	0
Peripheral nerves	1	1	1	0	0	0	0
Lumbar spinal cord	0	3	1	4	3	3	0
Thoracic spinal cord	0	7	10	10	8	1	0
Cervical spinal cord	0	1	3	1	2	0	0
Medulla oblongata	0	0	3	0	7	1	0
Cerebellum	0	0	1	2	3	0	0
Hemispheres	0	0	0	5	6	1	0
Bulbus olfactorius	0	0	0	0	3	1	0
Nasal cavity	0	0	0	0	0	0	0

The number of worms is the mean value (decimals values rounded off) from two infected mice.
*Only one mouse examined.
**Estimated number.

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Discussion

Localization in the nasal area of a host represents an unusual event in schistosomes, as in mammals only one species (*Schistosoma nasale*) occupies this site. However, little is known about the migration of *S. nasale* to this location. The same is true for avian schistosomes. *Trichobilharzia regenti* matures, mates and produces eggs in the duck nasal mucosa but prior to this schistosomula migrate through the spinal cord and brain in order to reach the final location (Horák *et al.*, 1998, 1999; Horák & Kolářová, 2001).

The present experiments have confirmed that *T. regenti* migrates via the nervous system and this agrees with the work of Horák *et al.* (1999). It appears that peripheral nerves (*n. ischiadicus* and *n. femoralis*) represent the main route for schistosomula to reach the spinal cord. Worms enter the spinal cord about 2 days p.i. in ducklings and mice. An alternative route might lead through the *arteria spinalis*. In the present experiments and those of P. Horák & J. Dvořák (unpublished) schistosomula were occasionally found in the lungs 2–3 days p.i. but not in the blood vessels and, therefore, we consider this blood migration to be of minor (or no) importance.

One aim of the present study was to compare the migration rate, location and number of worms in different organs and tissues of specific vs. nonspecific, immunodeficient vs. immunocompetent vertebrate hosts. Differences in the migration rates of worms in various hosts were evident during the first 3 days p.i. In this phase worms were recorded from the duck synsacral and thoracic spinal cord, whereas in all strains of mice worms reached the cervical spinal cord and the medulla oblongata and, in SCID mice, even the cerebellum. These migration rates are likely to be due to differences in the host body size. Subsequently, the migration of schistosomula in the BALB/c and hr/hr mice was approximately the same until day 9 p.i., whereas in SCID mice worms migrated faster and invaded the medulla oblongata and cerebellum before day 9 p.i. We hypothesize that the similarity of parasite migration in hr/hr and BALB/c mice may be influenced by their immunocompetence. As far as the later phase of infection (i.e. after day 9 p.i.) is concerned, the migration of schistosomula in BALB/c mice completed in the hemispheres where the worms appeared until day 24 p.i. In hr/hr mice worms did not migrate to the hemispheres and cerebellum but stopped in the medulla oblongata and spinal cord. However, P. Kouřilová (unpublished) found schistosomula of the same parasite in the brain of hr/hr mice but the reason for this is unknown. One explanation could be the low number of experimental hosts used. In the immunodefficient SCID mice, worm migration in the CNS was faster than in the other mouse strains and worms were found in the entire spinal cord, hemispheres and bulbus olfactorius. The location for maximum worm abundance was the synsacral spinal cord in ducks and thoracic spinal cord in mice; about 50% of the worms resided in the synsacral or thoracic spinal cord of hosts also during the later phases of infection and only about 50% of the worms continued in migration. The reason for this is unknown.

In the present experiments there appeared to be no relationship between the number of cercariae in the exposure dose and the number of worms actually penetrating the experimental hosts. Despite using freshly emerged cercariae, this effect may be influenced by differences in cercarial viability/infectivity in particular batches or be due to the ability of cercariae to respond to physical/chemical signals produced by the host. There was also no clear relationship between the developing symptoms in the experimental hosts and the number of migrating schistosomula. Exposure to cercariae was followed by clinically inapparent infections in all inbred mouse strains. These results differ from those of Horák *et al.* (1999) and P. Kouřilová (unpublished) who report leg paralysis in mice, suggesting that factors influencing the appearance of severe clinical symptoms require further clarification. On the other hand, leg paralysis and balance disorders were regularly observed in ducklings during the later phases of infection (7–18 days p.i.). Although location of schistosomula at days 6 and 9 p.i. in the spinal cord was similar, no leg paralysis was recorded during days 1–6 p.i. This suggests that the development of paralysis is probably time-dependent and influenced by progressive neuropathological changes in the spinal cord. Futhermore, the *in vitro* affinity of schistosomula for the peripheral nerves was confirmed, although the chemical signals attracting schistosomula to the nerves are unknown and should be considered in the future.

It can be concluded that *T. regenti* is a specific parasite of the nervous system. The majority of schistosomula older than 1 day are located in the nervous system, except for the worms being found in the lungs (tables 1 and 2). All other organs and tissues were parasite free. The present study has shown, for the first time, that schistosomula recognize peripheral nerves and use them to migrate to CNS. This represents a new type of migratory behaviour for schistosomes.

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