Original Article

Impact of Induced T_h1/T_h2 Shift on *Trichobilharzia regenti* Infection in Mice

(bird schistosomes / schistosomula migration / T_h1/T_h2 polarization / immunomodulation / CNS / neurotropic parasite)

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Abstract. Bird schistosomes parasitize mammals as non-specific hosts. Neurotropic *Trichobilharzia regenti* migrates extravasally *via* nervous tissue in experimentally infected mice. The majority of successfully penetrated larvae remain in the skin; the rest migrate through peripheral nerves to the spinal cord and brain. The potential of schistosomula to leave the skin and enter the central nervous system vary, and may be associated with T_h1/T_h2 polarization of the host cell immune response. The aim of the present study was to evaluate the impact of induced shift in polarization of cell immune response on the migration of *T. regenti* larvae in mammals. For this purpose, non-specifically immunomodulated mice were infected. The localization and abundance of schistosomula and associated histopathological changes were followed using routine histological techniques. Markers characterizing T_h1 and T_h2 systemic immune responses were followed using flow cytometry. The study revealed that the shift towards T_h1 response at the time of infection correlates with the speed and intensity of schistosomula migration towards the brain and with the severity of accompanying pathologies. This indicates increased health risks associated with *T. regenti* infection for mammals (potentially including human) with previously modulated cell immune response that may occur under natural conditions, e.g. due to the exposure to another infectious agent.

Introduction

Bird schistosomes undergo dixenic life cycle, usually with fresh water pulmonate snails as intermediates and waterfowl as definitive hosts. They cover eight genera with approximately 58 species, the vast majority of them with the life history typical of schistosomes, reflecting that of well-known human species. However, a unique strategy was described for *Trichobilharzia regenti*, the nasal bird schistosome with intravertebrate stages showing neurotropism and obligatory migration *via* peripheral and central nervous tissue (Horák et al., 1999; Hrádková and Horák, 2002).

Besides birds, cercariae of bird schistosomes also attack the skin of mammals including humans. Cercarial dermatitis, the defensive skin immune reaction that eliminates invading cercariae directly in the skin, has long been recognized as the only risk associated with human exposure to bird schistosomes. However, recent findings show that cercarial dermatitis develops as type I immediate hypersensitivity reaction concurrent with sensitization, and thus that the entrapment and destruction of penetrating cercariae usually occurs only after repeated contact with these larvae (Horák and Kolářová, 2001; Kouřilová et al., 2004a,b). Experimental studies of parasite interaction with unsensitized mammalian host not only showed weaker to none skin reaction, but also confirmed successful cercarial transformation into schistosomula, their further development, migration through several tissues/organs and feeding of host tissues, although resulting in death of immature worms and parasite life-cycle failure (e.g. Olivier, 1953; Bacha et al., 1982; Haas and Pietsch, 1991; Horák and Kolářová, 2000; Hrádková and Horák, 2002; Chanová et al., 2007; Lichtenbergová et al., 2011). Based on these studies performed with various bird schistosomes in several mammalian host species (including primates; Olivier, 1953), a similar situation is proposed, although not confirmed, in primarily infected humans. (e.g., Bayssade-Dufour et al., 2001, Horák and Kolářová, 2001).

Various studies showed the transient presence of bird schistosomula in mammalian tissues being associated

with more or less severe pathologies; the pathology caused by *T. regenti* is the most serious. The details were described in mice: first, an inflammatory reaction with oedema, vasodilatation and tissue infiltrates occurs in the skin. Subsequently, migrating schistosomula cause neuronal inflammation in deeper dermis and subcutis (Kouřilová et al., 2004b). Further migration through peripheral nerves, spinal cord and brain leads to an inflammatory cell reaction with granuloma formation at the sites of worms' location, along with increased proliferation and activation of astrocytes, as well as pathologic changes of adjacent neurons (Kolářová et al., 2001; Lichtenbergová et al., 2011). Clinical symptoms due to the central nervous system (CNS) invasion vary from minor to serious balance disorders and/or leg paralysis and death (Horák et al., 1999; personal observation). The development of neurological symptoms correlates with the number of schistosomula localized in nervous tissue (Lichtenbergová et al., 2011). The number of schistosomula that reach the nervous tissue depends, obviously, on the infection dose, but also on the host immune status. Studies of the schistosomula migratory pattern revealed the majority of successfully penetrating larvae remaining in the skin without further migration. Only a minor proportion of skin schistosomula undergo invasion of CNS; however, this number is significantly higher in immunosuppressed (SCID) than in immunocompetent (SKH1 hr/hr) mice infected with the same dose of cercariae (Kouřilová et al., 2004a,b).

Efficient entrapment of penetrating worms in the skin of a sensitized immunocompetent host with cercarial dermatitis is attributed to T_h2 polarization of the immune response (CD3-deficient SCID mice do not develop this skin reaction; Kouřilová et al., 2004a,b). The response during the initial phase of primary infection, when schistosomula are able to leave the skin, was characterized by the same authors as T_h1/T_h2 mixed. This led us to the hypothesis that the change of the host immunological status (particularly the shift towards the T_h1 cell immune response) before or during exposure to migrating schistosomula will also influence the course of infection in the immunocompetent host.

Thus, the aim of the present study was to evaluate the impact of induced shift in the polarization of systemic cell response on the course of *Trichobilharzia* infection in mammals. For this purpose, the migratory pattern and development of schistosomula-associated pathologies were studied in BALB/c and C57BL/6 mice exposed to *T. regenti* cercariae after previous non-specific T_h1 cell immune response inducer application. Higher speed and intensity of migration to CNS was confirmed.

Material and Methods

Experimental models

Bird schistosomes of *Trichobilharzia regenti* are kept in complete laboratory cycle using snails *Radix lagotis* and ducks *Anas platyrhynchos* f. domestica as intermediate and definitive hosts, respectively. All the experiments were performed with adult females of BALB/c and C57BL/6 mice (Charles River Laboratories, Inc., Wilmington, MA) and freshly shed cercariae of *T. regenti* in the following pattern (overviewed in Table 1):

Group I – Immunostimulated infected mice: mice were subcutaneously injected with 100 μl of Complete Freund's Adjuvant (CFA, #F5881 Sigma, St. Louis, MO) and subsequently, 7 days post application, they were routinely exposed to cercariae (40 minutes of feet and tail immersion into a beaker containing approximately 2000 cercariae in 50 ml of tap water).

Groups II–IV – Experimental conditions followed those for Group I, except for physiological saline application instead of CFA (Group II – Non-stimulated infected mice); cercariae-free tap water exposure instead of water containing cercariae (Group III – Immunostimulated non-infected mice) and both, physiological saline application and cercariae-free tap water exposure instead of CFA and cercariae, respectively (Group IV – Control non-stimulated and non-infected mice).

Mice were sacrificed by overdosed anaesthesia (ketamine/xylazine) 3–14 days post infection (p.i.). All the experiments were consistent with current animal welfare laws of the Czech Republic and EU, and were approved by the Animal Experiment Board at Charles University in Prague.

Tissue processing

Heart, lungs, liver and kidney were extracted and tested for the parasite presence on fresh squashed slides using routine light microscopy. Spinal cord and brain were processed for histology (fixed in Bouin's fixative, paraffin embedded, cut into 4 μm sections and haematoxylin/eosin or Luxol fast blue/neutral red stained). Whole heparinized blood of a single mouse per particu-

Table 1. Overview of experimental model groups

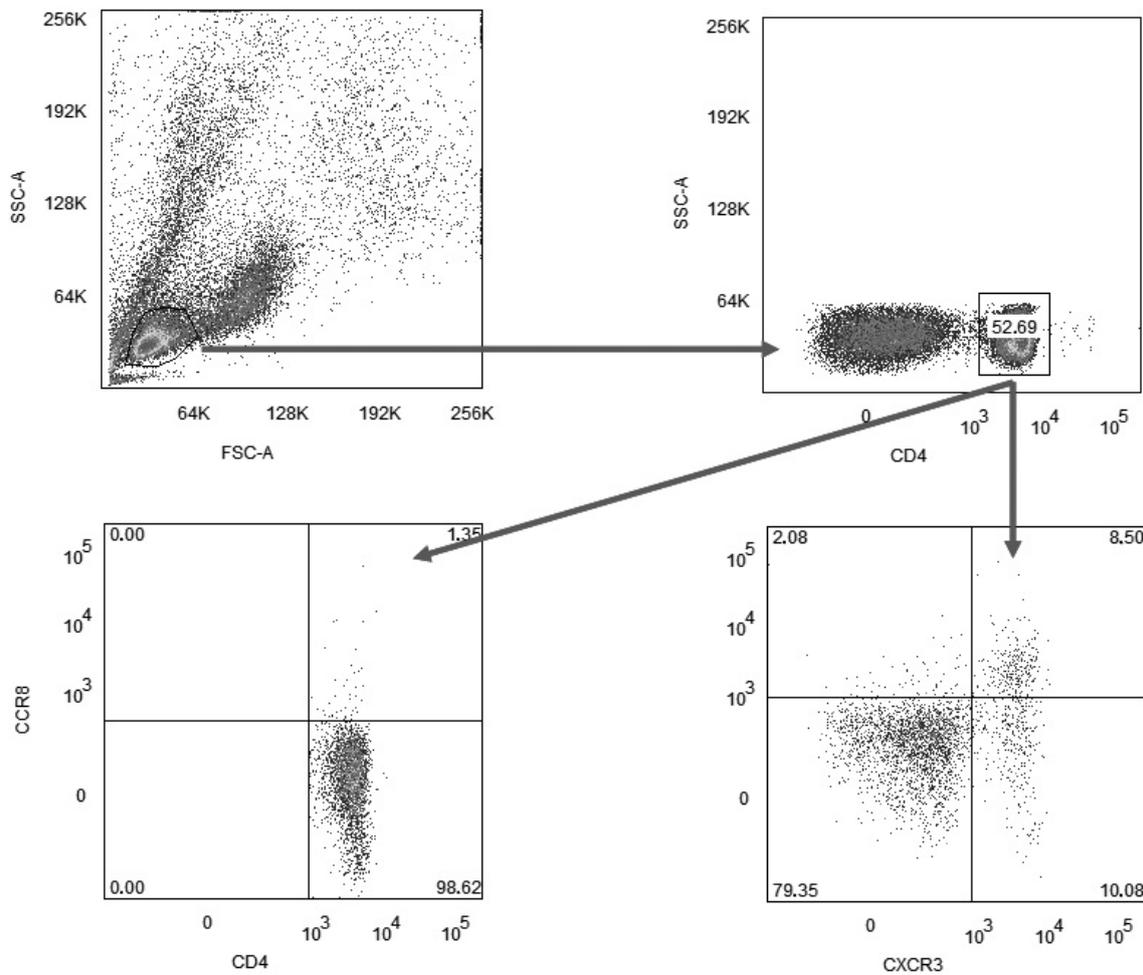| Group number	Group I		Group II		Group III		Group IV	
Description	Immunostimulated and infected mice		Non-stimulated infected mice		Immunostimulated non-infected mice		Control non-stimulated and non-infected mice	
Mouse strain	**BALB/c**	**C57BL/6**	**BALB/c**	**C57BL/6**	**BALB/c**	**C57BL/6**	**BALB/c**	**C57BL/6**
CFA injection	+	+	-	-	+	+	-	-
***T. regenti* infection**	+	+	+	+	-	-	-	-
Number of mice	15	15	15	15	3	3	3	3

Fig. 1. Typical flow cytometry data for the presence of chemokine receptors with gating strategy; Group I BALB/c mouse 7 days p.i.

lar experimental group and period was processed for flow cytometry.

Flow cytometry (FACS)

Heparinized whole blood was incubated with the following fluorescent antibodies: Allophycocyanin/Cyanine 7-conjugated anti-mouse CD4, #100414; phycoerythrin-conjugated anti-mouse CCR5, #107006; Peridinin-chlorophyll-protein/Cyanine5.5-conjugated anti-mouse CXCR3, #126514 – all supplied by BioLegend Inc., San Diego, CA and fluorescein isothiocyanate-conjugated anti-CCR8, #ABIN732870 Antibodies-online Inc., Atlanta, GE. Subsequently, red blood cells were lysed (BD FACS™ Lysing Solution, #349202, BD Biosciences, San Jose, CA), the samples were washed 3x with phosphate-buffered saline and immediately acquired using BD FACS Canto II (BD Biosciences). At least 100,000 events per sample were recorded. Data were evaluated using FlowJo (TreeStar Inc., Ashland, OR). Firstly, the lymphocyte population was gated and then CD4⁺ T cells were selected for further detailed analyses for the presence of CXCR3, CCR5 and CCR8 chemokine receptors (see gating strategy in Fig. 1). Relative numbers of cells with particular surface marker were acquired (reported as percent of CD4+ cells in the blood sample). Through-

out the text, CXCR3⁺ and CCR5⁺ cells are considered T_h1, and CCR8⁺ cells are considered T_h2, based on typical expression of chemokine receptors on these cells (e.g. Saxena et al., 2012).

Results

T_h1 / T_h2 balance

The counts of cells labelled with anti-CXCR3, anti-CCR5 and anti-CCR8 antibodies differed between Group III and Group IV mice on the day of exposure to *T. regenti* larvae (day 0; 7 days post CFA/saline injection; Fig. 2). Particularly, the percentage of CXCR3⁺ cells was higher, and the percentage of CCR8⁺ cells was lower in CFA-injected mice of both strains than in the corresponding controls. The percentage of CCR5⁺ cells in CFA-injected mice was higher only in BALB/c and equal in C57BL/6 compared to controls. The highest difference between CFA-injected and control mice was observed for CXCR3⁺ cells (approximately 4 % for both mouse strains). Among all mouse groups, the highest number of T_h1 cells on the day of infection was measured in CFA-injected BALB/c mice. The highest num-
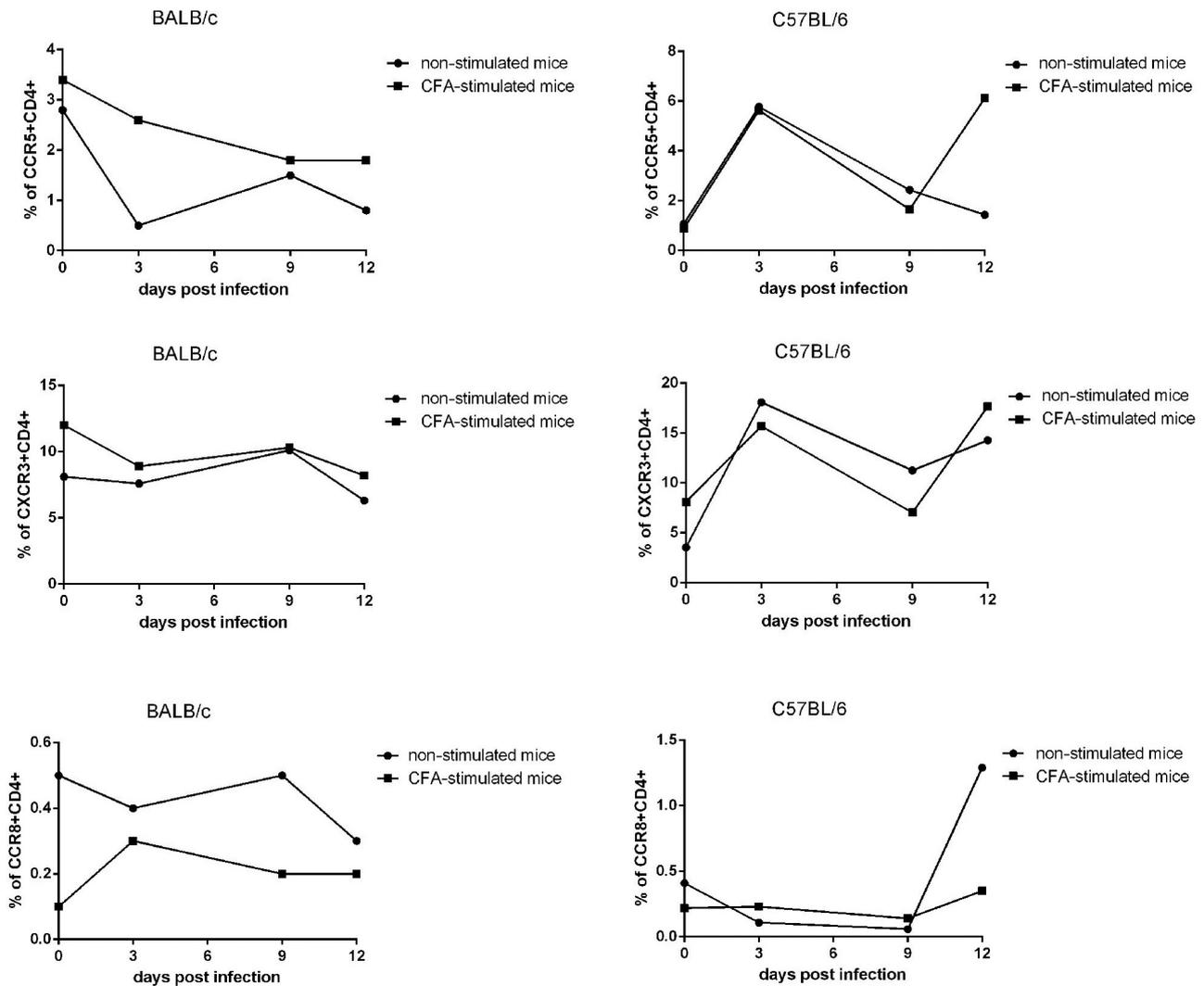
Fig. 2. Presence of chemokine receptors typical of T_h1 (CXCR3, CCR5) and T_h2 (CCR8) cells in CFA-immunostimulated and non-stimulated mice of BALB/c and C57BL/6 strains in different periods post infection with *T. regenti*. Flow cytometry analysis

ber of T_h2 and the lowest number of T_h1 cells were seen in control BALB/c mice at the same time.

Shifts in the ratio of particular CD4+ cells in the next 14 days after infection differed between the mouse groups and strains:

In infected BALB/c mice previously injected with CFA (Group I), the initial situation with more T_h1 and less T_h2 cells compared to non-injected (Group II) mice remained in existence also after the infection. Particularly, the relative number of CCR8+ cells was lower in Group I than in Group II mice and the relative number of both, CXCR3+ and CCR5+ cells, was higher in Group I than in Group II mice at corresponding time intervals during the entire period under study. Nevertheless, the number of T_h1 cells was continually decreasing in both groups.

In C57BL/6 mice, the initial situation switched soon after the infection. The relative number of CXCR3+ cells in Group II mice exceeded that of Group I and the relative number of CCR8+ cells in Group II mice declined below the count in Group I mice; almost no difference in

CCR5+ cell counts was measured between both groups after the infection. In both C57BL/6 mouse groups, the number of T_h1 cells exceeded the initial maximal numbers soon after infection.

Schistosomula localization and invasion of CNS

No worms were detected in visceral organs of any mouse tested. The first schistosomula in the spinal cord were detected 3 days p.i. in all groups of infected mice. In most mice, schistosomula were also observed in *medulla oblongata*. A few worms (usually 1–3 per mouse) were found in Group II BALB/c mice at this period, contrary to other groups (Group I BALB/c with 5–10, and both, Group I and II C57BL/6, with more than 10 schistosomula per mouse). Initially, schistosomula appeared in white matter (Fig. 3a), and subsequently also in grey matter (Fig. 3b); earliest spread to the grey matter occurred in Group II BALB/c mice. Intact schistosomula were present in the spinal cords of all mice for the rest of the period under study, with highest numbers recorded in Group I BALB/c mice 5 days p.i. Although
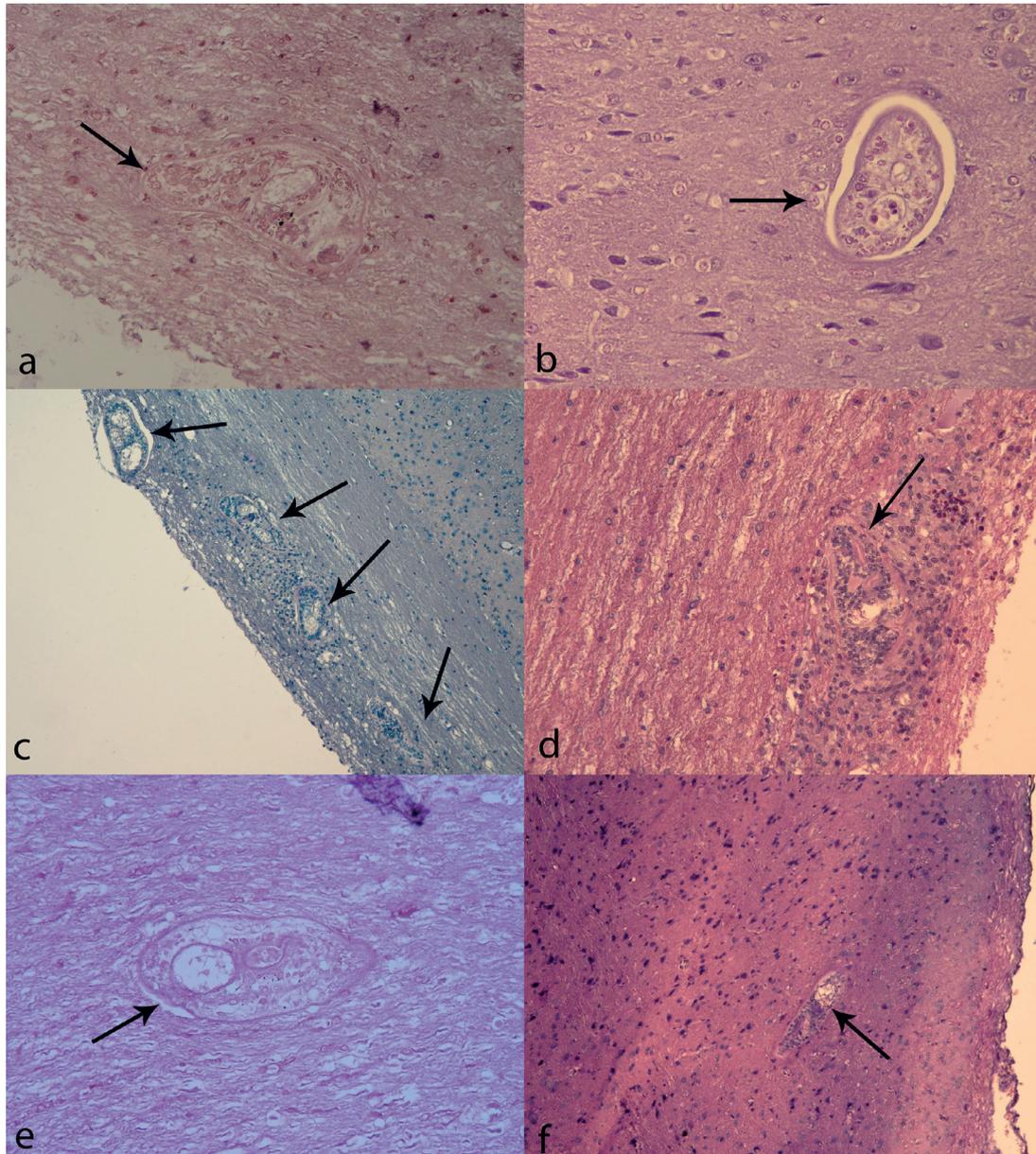
Fig. 3. Invasion of murine CNS by schistosomula of *Trichobilharzia regenti* with or without previous non-specific immunomodulation with CFA. Haematoxylin/eosin staining

a – schistosomulum in the white matter of spinal cord; Group II C57BL/6 mouse 3 days p.i; **b** – schistosomulum in the grey matter of spinal cord; Group II BALB/c mouse 7 days p.i; **c** – massive infiltrates composed mainly of macrophages, lymphocytes and eosinophils surrounding accumulated schistosomula in the spinal cord; Group I BALB/c mouse 5 days p.i; **d** – infiltrates surrounding individual schistosomulum in the spinal cord; Group II BALB/c mouse 8 days p.i; **e** – schistosomulum in the spinal cord – note the absence of inflammatory cells; Group I C57BL/6 mouse 9 days p.i; **f** – schistosomulum in the brain hemisphere; Group I BALB/c mouse 8 days p.i.; **arrows** – schistosomula.

the number of larvae decreased with time, the differences in the intensity of spinal cord invasion in different mouse groups remained clear until the end of the study. The first worms in the brain were detected 7 days p.i. in Group I BALB/c mice, 9 days p.i. in Group I C57BL/6 mice and as late as 11 days p.i. in Group II mice of both strains. The number of worms found in the brain did not exceed three per mouse. Schistosomula were found mostly in the *pons*, *cerebellum* and *pia mater*; rather random schistosomula distribution in the brains of all mouse groups was observed. For a review of the speed

and intensity of CNS invasion in different mouse groups, see Table 2.

The presence of schistosomula was associated with infiltration of immune cells (mainly macrophages, lymphocytes and eosinophils) in BALB/c, but rarely in C57BL/6 mice. In BALB/c mice, the extent of infiltration seemed to reflect the schistosomula number: substantial granulomas were formed around the accumulated worms as well as in their migratory route traces in Group I BALB/c mice from day 5 p.i. (Fig. 3c); less extensive infiltrations surrounding isolated larvae in

Table 2. Localization of *T. regenti* schistosomula in CNS of non-stimulated (Group II) and CFA-injected (Group I) BALB/c and C57BL/6 mice 3–11 days post infection (dpi; 3 mice for each group and period were investigated)

dpi	Group II BALB/c spinal cord & *medulla oblongata*	brain	Group I BALB/c spinal cord & *medulla oblongata*	brain	Group II C57BL/6 spinal cord & *medulla oblongata*	brain	Group I C57BL/6 spinal cord & *medulla oblongata*	brain
3 dpi	+ (wm)	-	++ (wm)	-	+++ (wm)	-	+++ (wm)	-
5 dpi	+ (wm)	-	+++ (wm)	-	++ (wm)	-	N/A	N/A
7/8 dpi	+ (wm)	-	+++ (wm, gm)	+	++ (wm)	-	++ (wm)	-
9 dpi	+ (wm, gm)	-	++ (wm, gm)	+	++ (wm, gm)	-	++ (wm)	+
11 dpi	+ (wm)	+	++ (wm, gm)	+	+ (wm, gm)	+	++ (wm)	+

+ 1–5 schistosomula; ++ 5–10 schistosomula; +++ > 10 schistosomula; N/A – data not available; wm – white matter; gm – grey matter.

Group II BALB/c mice appeared three days later (Fig. 3d). Contrary to this finding, only a weak influx of inflammatory cells was seen in Group II C57BL/6 mice from day 9 p.i. and almost no cellular reaction was recorded in Group I C57BL/6 mice (Fig. 3e). No cellular reaction was observed in the brains throughout the investigated mouse groups (Fig. 3f). Luxol fast blue staining for myelin did not reveal any demyelination in any specimen.

Discussion

Trichobilharzia regenti was described in 1998 (Horák et al., 1998), and establishment of the laboratory life cycle followed. Since that time, host-parasite interactions of *T. regenti* with immunocompetent murine hosts has been studied using several mouse strains (BALB/c, C57BL/6 and SKH1 hr/hr), with details on the migration and pathogenesis described for BALB/c mice only. Mice of the C57BL/6 strain were used in the studies of the skin phase focused on cercarial dermatitis, and no details on the migration to CNS were provided. In the present study, the impact of cell immune response polarization on the infection cruise was in focus. Therefore, mice of T_h1 dominant C57BL/6 and T_h2 dominant BALB/c strains (Watanabe et al., 2004) were used in order to compare schistosomula migration in mice with differently polarized response, either due to previous immunomodulation or genetic predisposition.

To confirm the differences in T-helper cell counts among mouse groups before and during the infection, flow cytometry analysis was performed. Use of CCR5, CXCR3 and CCR8 chemokine receptors as surface markers for T-helper cell subsets was based on their typical expression on T_h1 and T_h2 cells, respectively. Although the employment of chemokine receptor presence for phenotyping T-helper cells has been debated (e.g. Chiu et al., 2002), the authors believe that their use in the present study fulfils its objective.

T. regenti is kept in the laboratory cycle running for several years. Migration in mice was described in detail in the last decade and authors are familiar with its obligatory pattern. However, variability in the penetration success, survival in mammalian tissues, speed and intensity of CNS invasion, and severity of associated pathologies have been reported since the species description in 1998 (Horák et al., 1998). The ability to parasitize a host depends on the particular mouse strain used, but also seems to be influenced by long-term passage in the laboratory life cycle (personal observation). The latter should be taken in consideration when comparing the above-mentioned variables (especially the exact worm numbers detected in selected location or infection phase) with published data. Therefore, here reported results referring schistosomula quantity were compared with controls from the present study only. The route and timing of migration are discussed with all the data available.

CFA is a strong T_h1 inducer (Jensen et al., 1998). Its application in the present study showed a desired effect – change in T_h1 cells' relative counts 7 days post injection in mice of both strains (more significant in BALB/c than C57BL/6 mice).

The migratory route of schistosomula reported here did not show any dissimilarities either among the mouse groups tested in the present study, or in comparison with the published data (Hrádková and Horák, 2002; Lichtenbergová et al., 2011). The time frame of the migration and number of schistosomula detected in various locations/periods, however, differed significantly among the tested mouse groups.

The period of first schistosomula detection in the spinal cord and *medulla oblongata* reported in the present study (3 days p.i. for all mouse groups) is in agreement with both the published data and the long-term experience of the first author (the earliest finding in spinal cord 2 days p.i. was reported, e.g. by Hrádková and Horák (2002)). The period of first schistosomula occurrence in the brain (minus the above-mentioned *m. oblongata*) differed among the mouse groups in the present study (7 to 11 days p.i. in particular groups). According to published data, the first schistosomula reach the brain around day 11 p.i.; the same timing was observed for Group II BALB/c mice in the present study. A regular presence of worms in the brain as soon as in Group I BALB/c mice described here has not been reported yet.

The speed and intensity of migration seems to correlate with the relative numbers of investigated CD4+ cells. Comparing all mouse groups, the highest number of T_h1 cells was detected in CFA-injected BALB/c on the day of infection, and was exceeded in C57BL/6 mice

of both groups soon after the infection. The number of schistosomula that had reached CNS in these mice (Group I BALB/c, Groups I and II C57BL/6) was significantly higher than in non-stimulated (Group II) BALB/c mice.

The differences in speed of further schistosomula migration were also obvious. The difference between Group I and Group II BALB/c mice was substantial, with faster establishment in the brain of CFA-injected BALB/c mice. In contrast, differences in the speed among two groups of C57BL/6 mice were minor and in both groups, schistosomula appeared in the brain sooner than in Group II BALB/c mice. We believe that the higher intensity and speed of migration from the skin to CNS was caused by the initial rise in T_h1 cell numbers.

The number of T_h1 cells was continually decreasing in BALB/c mice, but increasing in C57BL/6 mice after infection, probably due to their different predisposition to T_h1/T_h2 response development. A strong T_h1 response in C57BL/6 mice may have prevented granuloma formation in spite of numerous schistosomula present in the spinal cord. In contrast, the reaction in BALB/c mice was obvious and its intensity correlated with the schistosomula number, as previously reported by Lichtenbergová et al. (2011). Thus, infection with *T. regenti* resulted in most significant pathologies in immunomodulated (CFA-injected) BALB/c mice. This finding supports our hypothesis that the shift in polarization of the cell immune response increases the risks associated with *T. regenti* infection in mammals.

Bird schistosomes of the genus *Trichobilharzia* with more than 40 species occur worldwide and still spread over formerly unaffected areas. Human exposure to bird schistosomes manifested with cercarial dermatitis is frequently reported from many regions, including those in temperate and cold zones, and has been recently described as a re-emerging disease (de Gentile et al., 1996; Horák and Kolářová, 2011). When speculating about the possible schistosomula survival in human, CNS involvement is unlikely, mainly due to the host body size with distances invincible for these worms. However, entering the peripheral nerves and induction of local inflammatory response close to the penetration site seems realistic. Although no exact evidence on the migration in human has been provided (possibly due to the lack of a useful diagnostic tool according to Horák et al., 2008), several studies reviewed by these authors pointed to the necessity to also consider schistosomula-associated injuries. The present study even enhances this suggestion.

In conclusion, our results show that the rise in T_h1 cell counts in initial phase of *T. regenti* infection is associated with faster and more intensive migration towards CNS. We therefore suppose that natural conditions influencing the T_h1/T_h2 balance, e.g. previous or simultaneous contact with other infectious agents, may also act and significantly increase the risks connected with the exposure to bird schistosomes. This should also be kept in mind in human cases with history of contact with these parasites.

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