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# *Trichobilharzia regenti*: Antigenic structures of intravertebrate stages

**Communication**

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**Abstract:** Like several other bird schistosomes, neurotropic schistosome of *Trichobilharzia regenti* can invade also mammals, including humans. Repeated infections cause cercarial dermatitis, a skin inflammatory reaction leading to parasite elimination in non-specific mammalian hosts. However, in experimentally primo-infected mice, the worms escape from the skin and migrate to the central nervous system. In order to evade host immune reactions, schistosomes undergo cercaria/schistosomulum transformation accompanied with changes of surface antigens. The present study is focused on localization of the main antigens of *T. regenti*; cercariae, schistosomula developed under different conditions and adults were compared. Antigens were localized by immunofluorescence and ultrastructural immunocytochemistry using sera of mice repeatedly infected with *T. regenti*. Detected antibody targets were located in glycocalyx and penetration glands of cercariae and in tegument of cercariae, schistosomula and adults. Shedding of cercarial glycocalyx significantly reduced surface reactivity; further decrease was reported during ongoing development of schistosomula. Spherical bodies, probably transported from subtegumental cell bodies to worm surface, were identified as the most reactive tegumental structures. Based on similar results for schistosomula developed in specific, non-specific hosts and *in vitro*, it seems that the ability of *T. regenti* to decrease the surface immunoreactivity during ontogenesis is independent on the host type.

**Keywords:** *Trichobilharzia regenti • Neurotropic schistosome • Immunoreactivity • Immunolocalization • Ultrastructure*



## 1. Introduction

*Trichobilharzia regenti* is a bird schistosome able to infect mammals as non-specific (accidental) hosts. Schistosomula of this species, contrary to all other schistosomes with known life cycles, use the nervous tissue for migration within the host body of both, avian and mammalian hosts. The infection is commonly manifested by neuromotor disorders like hind leg paralysis [1]. In experimentally primo-infected mice, cercariae completely transform to schistosomula, find the peripheral nerves and migrate within the nerve fascicles or in the *epineurium* to the spinal cord and brain. Migrating schistosomula feed on the surrounding nervous tissue [2]. Due to the host-parasite incompatibility, the migration and development in mice remain incomplete and parasites die within certain period post infection (p.i.) [3].

Repeated infections of mammals lead to the development of skin immune response manifested as cercarial dermatitis, which is sufficient for capture and destruction of cercariae in the skin. This allergic skin reaction is Th2–associated, and was described as an early type I immediate hypersensitivity response followed by a late phase of cutaneous inflammatory reaction against the penetrating larvae [4]. Glycocalyx as well as E/S products of cercariae seem to contain major antigens capable to induce a strong antibody response. Especially, a 34 kDa protein from cercarial homogenate and E/S products is recognized by mouse

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IgG and IgE and probably participates in the induction of Th2 immune response [5]. However, details on localization of this antigen are still lacking.

The surfaces of cercariae and schistosomula are immunologically important structures. Cercarial glycocalyx of both human and bird schistosomes is recognized by antibodies from humans and laboratory rodents infected with *Schistosoma mansoni*, *Trichobilharzia szidati* and *T. regenti* [6]. Therefore cercaria/schistosomulum transformation accompanied by loss of glycocalyx and formation of a heptalaminate surface membrane is believed to be involved in avoiding antibody- and complement-mediated damage by the host immune system [7,8]. Studies on human schistosomes showed that early skin-stage schistosomula are susceptible to humoral and cellular host immune responses [9], which is illustrated by their relative sensitivity to both complement-mediated killing and antibody-dependent cellular cytotoxicity. Later stages, *i.e.* lung schistosomula and adult worms become resistant to both, humoral and cellular immune responses [10,11]. Some of the evasion mechanisms such as antigenic surface mimicry, membrane turnover, modulation of expression of surface antigens and immunomodulatory role of surface proteins have been suggested by Abath and Werkhauser [12]. The tegument of schistosomes is believed to be a dynamic structure crucial to the parasite survival in terms of immune evasion (for review see [13]). In the case of human schistosomes, identification of accessible proteins in the tegument is an important step for selection of target molecules for vaccines and drugs [14].

Contrary to the extensive studies on blood-dwelling human schistosomes, knowledge of the mechanisms of immune evasion employed by bird schistosomes remains poor. *Trichobilharzia regenti* may employ adaptations, including immune evasion strategies, distinct from those used by human and bird visceral schistosomes, due to highly specific migration through different environments within the host body (skin, CNS, nasal tissue). Until now, no clear data are available on the role of mammalian immune response in the elimination of developing worms and accessibility of the antigenic target molecules during parasite development. Therefore, we focused on localization of worm antigens which trigger production of mouse antibody. Sera from mice repeatedly infected with *T. regenti* were used for detection of immunoreactive structures of cercariae, schistosomula (developed in specific or non-specific hosts and under *in vitro* conditions) and adult worms matured in ducks. The immunoreactive structures were characterized using immunofluorescence (IF) and ultrastructural immunogold labeling.

# 2. Experimental Procedures

## 2.1 Parasites

Cercariae, 3 and 5 days old schistosomula developed in birds and mice, adult worms developed in birds, and 1, 3 and 5 days old schistosomula cultivated *in vitro* were used. Cercariae were released from the snail intermediate hosts (*Radix lagotis*; maintained under laboratory conditions) into tap water. For embedding, cercariae were concentrated employing their positive phototaxis and subsequently immobilized by cooling on ice. For transformation and development of schistosomula *in vivo*, the hosts were exposed to approx. 2000 cercariae for 45 minutes. Ducks (*Anas platyrhynchos* f. domestica; 1 week old ducklings) and mice (ICR; 2 months old) were infected as described by Horák *et al*. [1]. The animals were sacrificed by decapitation (ducks) or cervical dislocation (mice) on days 3 and 5 p.i. for collection of schistosomula, eventually 18 days p.i. (ducks only) for collection of adults. Spinal cord and nasal tissue were excised for isolation of schistosomula and adult worms, respectively, placed into saline buffered with 20 mM Tris-HCl pH 7.8 and torn by needles. Released worms were picked up, repeatedly washed with fresh buffer and concentrated by centrifugation. For *in vitro* cultivation, concentrated cercariae were processed as previously published [15] and then placed into SCM 169 cultivation medium supplied with 0.5% Penicillin/Streptomycin/Amphotericin B (100X; BioWhittaker 17-745E). Schistosomula were collected from the medium after desired period post transformation (p.t.) and repeatedly washed in fresh buffer.

## 2.2 Sample processing

For IF, cercariae, schistosomula and adult worms were fixed with Bouin's fixative, embedded in JB-4 Plus® (Polysciences), and sectioned to 2 µm. For transmission electron microscopy (TEM), the samples were processed by cryofixation using high pressure freezing (Leica EM PACT2) and subsequent cryosubstitution (Leica EM AFS) terminated with embedding in LR White™ resin (Polysciences), ultrasectioned (80 nm) with ultramicrotome Ultracut E (Reichert-Jung) and placed on uncoated nickel grids.

## 2.3 Immunization of mice

Four mice (C57BL/6; 2 months old) were immunized by repeated exposure to freshly emerged *T. regenti* cercariae (approx. 2000 per infection per mouse) on days 0, 10, 20 and 30. One control mouse remained uninfected. Mice were bled for serum extraction 10 days after the 4$^{th}$ infection (on day 40). Sera were separated by

repeated centrifugation (3500 rpm, 3x10 min.), aliquoted and frozen. Reactivity of sera with the homogenate of *T. regenti* cercariae was tested by ELISA as described previously [5].

### 2.4 Immunohistochemistry

For IF, sections were blocked with 2% goat serum and 1% BSA (30 min), incubated with immune or negative sera (dilution 1:150; 60 min), and the reaction was detected by Alexa Fluor® 488 goat anti-mouse IgG secondary antibody (Invitrogen; dilution 1:1000; 30 min). The sections were mounted using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Inc.). Haematoxylin/eosin (H/E) staining of corresponding sections was performed when desirable, after inspection under the fluorescence microscope (Olympus BX51).

For TEM, sections on nickel grids were incubated with immune or negative mouse sera (dilution 1:400; 60 min) and the bound antibody was visualized by reaction with 10 nm colloidal gold-labeled goat anti-mouse IgG secondary antibody (Sigma; dilution 1:300; 60 min). Sections were finally post-fixed with 2% glutaraldehyde in 0.1M phosphate buffer and examined using a transmission electron microscope (JEOL 1011).

All experiments and the maintenance of experimental animals were consistent with current animal welfare laws of the Czech Republic and were approved by the Animal Welfare committee of Charles University.

## 3. Results and Discussion

The present study was a screening for immunoreactive structures of three different life stages of *T. regenti*. Cercaria represents the infective stage interacting with mouse immune system while penetrating the skin. Schistosomulum is in a close contact with the immune system during migration through the host body; comparison of immunoreactivity of three groups of schistosomula should reveal potential variability in antigen expression by parasites developed under different conditions (duck, mouse, artificial medium). Adults occur within the specific host in a different location than schistosomula [16]. By testing the mouse sera reactivity with adult worms we showed further developmental changes associated with distribution of antigens.

Prior being used for immunolocalization, all sera of the re-infected mice were tested by ELISA. Significantly increased level of IgG antibodies against the homogenate of *T. regenti* cercariae was detected and no reaction of the negative mouse serum was observed. Two approaches (IF and TEM) were used for immunolocalization of antigens, and comparable results were obtained. Only in the case of cercarial penetration glands and the tegument of adults, the antigens were localized by immunogold labeling but not by IF. We suppose that the use of cryofixation for TEM sample processing may lead to a better preservation of antigenic epitopes and make this method more sensitive.

For overview of antibody binding detected by IF see Table 1. All mouse sera reacted with the surface of cercariae (Figure 1a), and the tegument and subtegumental structures of schistosomula (Figure 1b-d). No reaction with adults was observed by means of IF. Immunoreactive subtegumental structures differed in localization in schistosomula of different age. In 1 day old schistosomula, the recognized structures were aggregated dorsally, approximately in the mid body region (Figure 1b). In 3 and 5 days old schistosomula, the structures were restricted to bordered clusters found in the parenchyma (Figure 1c, d). Similar distribution

| | Stage | Surface | Penetration glands | Intestine | Subtegumental structures / Parenchyma |
|---|---|---|---|---|---|
| | Cercariae | +++ | - | - | - |
| | 1 day *in vitro* | ++ | - | - | +++ |
| Schistosomula | 3 days *in vitro* | + | X | - | +++ |
| | 3 days - duck host | + | X | - | +++ |
| | 3 days – mouse host | + | X | - | +++ |
| | 5 days *in vitro* | (+) | X | - | +++ |
| | 5 days - duck host | (+) | X | - | +++ |
| | 5 days – mouse host | (+) | X | - | +++ |
| | Adults | - | X | - | - |

**Table 1.** Immunofluorescent reaction of different *T. regenti* life stages with sera of mice 4-times infected with *T. regenti* cercariae. (+) / + / ++ / +++ increasing intensity of reaction; - no reaction detected; X indicates the absence of structure in particular stage.

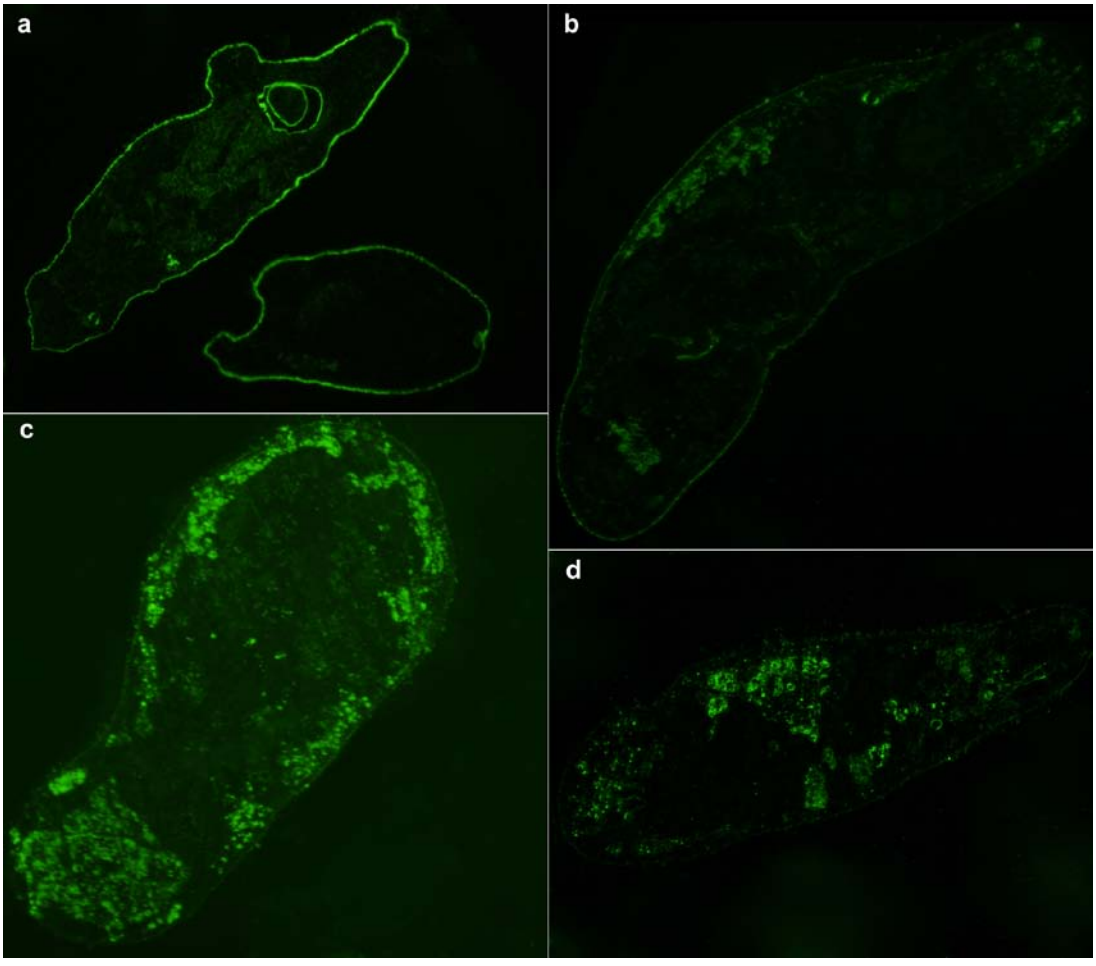


**Figure 1.** Reactivity of *T. regenti* cercariae and schistosomula with sera of 4-times re-infected mouse (Alexa Fluor® 488). a – cercaria; strong reaction with the surface; b – 1 day old schistosomulum developed *in vitro*; reaction with subtegumental structures aggregated above the acetabulum; reaction with the surface; c – 3 days old schistosomulum developed *in vitro*; reaction with subtegumental structures dispersed in parenchyma and weak reaction with the surface; d – 5 days old schistosomulum developed in duck; reaction with subtegumental structures and weak reaction with the surface. Note the changes in the distribution of immunoreactive subtegumental structures and decreased reactivity of surface during development.

of these clusters was observed in schistosomula of the same age developed under different conditions (in duck or mouse host, in the culture). Counterstaining with DAPI and staining with H/E did not show the structures to be associated with any compact tissue/organ. Mostly, discrete foci surrounding nuclei of cells in subtegumental area were observed. Control serum from the unexposed mouse did not exhibit reaction with any worm structure.

Reaction of sera with sections for TEM confirmed stage-specific distribution of the recognized antigens. In cercariae, the most intensive specific IgG reaction occurred with glycocalyx (Figure 2a); cercarial glycocalyx was proven to bind the antibody to a larger extent than any structure of the other stages. The remnants of cercarial glycocalyx still present on 1-day old schistosomula transformed *in vitro* were labeled with specific antibody, too (for description of *T. regenti* development *in vitro* see [15]). This agrees with the results of Kouřilová *et al*. [6]. As previously postulated for human schistosomes [17], cercarial glycocalyx is a major immunogen and activator of the alternative pathway of complement and, therefore, shedding of glycocalyx represents an important evasion strategy for survival in the hostile environment of the definitive host. Disappearance of lectin and antibody targets from the surface of schistosomula was also confirmed after cercaria/schistosomulum transformation of another bird schistosome, *T. szidati* [8].

The content of post- and circum-acetabular penetration glands of cercariae and 1 day old schistosomula showed only a weak reaction which was detected just by immunogold labeling (not illustrated). No reaction of penetration glands was detected in a similar study with the visceral species *T. szidati* [8].

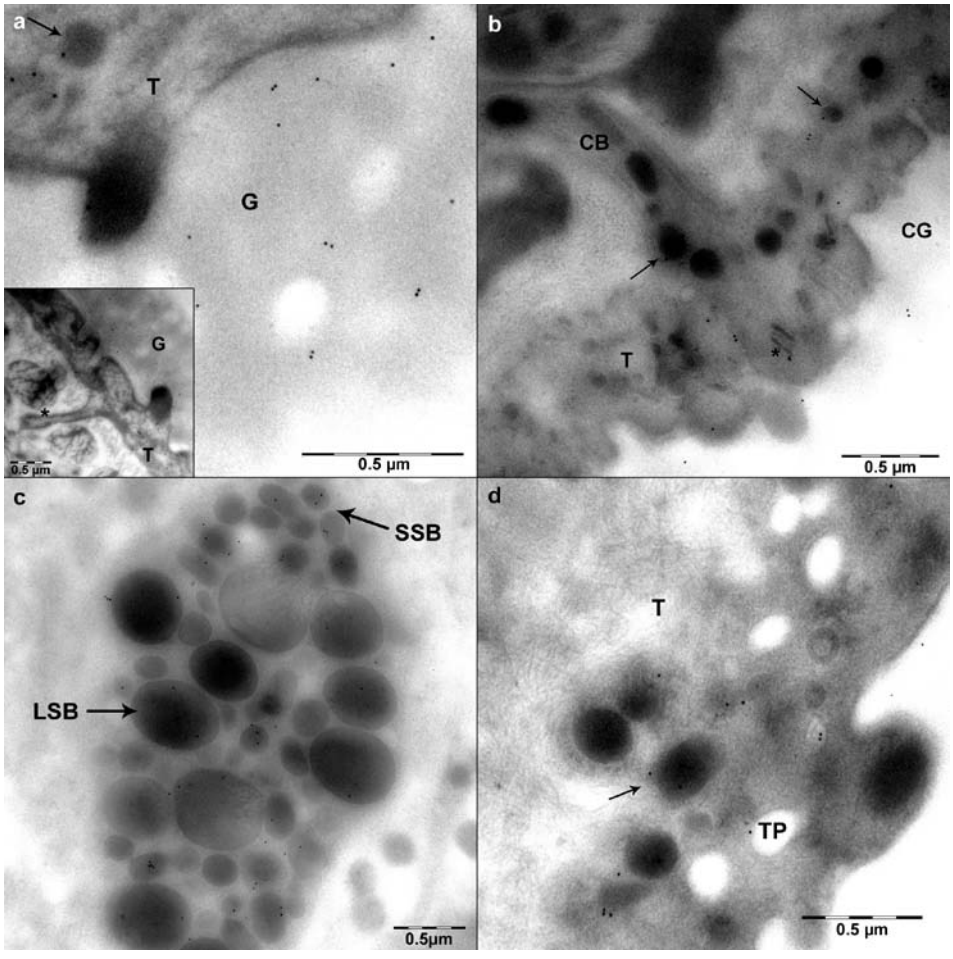


**Figure 2.** Structures of *T. regenti* recognized by sera of 4-times re-infected mouse. Immunogold labeling method for TEM using 10 nm colloidal gold-labeled secondary antibody. a - glycocalyx (G) and apical tegumental syncytium (T) with spherical body (arrow) of cercaria recognized by mouse antibodies; note empty cytoplasmic bridge marked with asterisk at small picture; b - apical tegumental syncytium (T) with cytoplasmic bridge (CB) and remnants of cercarial glycocalyx (CG) recognized by mouse antibodies; antibody binding to spherical (arrow) and elongate bodies (asterisk) of 1 day old schistosomulum developed *in vitro*; c – subtegumental cell with two types of spherical bodies (small – SSB and large – LSB) recognized by antibodies, 3 days old schistosomulum developed in mouse; d – antibody labeling of spherical bodies (arrow) and membrane of tegumental pits (TP) in tegumental syncytium (T) of 3 days old schistosomulum developed in duck.

Nevertheless, the sole use of IF could let the weak reaction unrevealed. Cercarial secretory products released from the penetration glands are essential for glycocalyx shedding and parasite penetration through the host tissues [18,19], and molecules contained in these products represent immunogens able to induce a strong antibody response and bias the immune response to Th2 polarization [5]. Cercarial glands occupy approximately one third of cercarial body volume and their products consist of a large amount of material [20], nevertheless, the molecules acting as immunogens may represent a small portion of cercarial gland content. This could explain only sporadic binding of antibody.

The other immunoreactive structures detected in cercariae were spherical bodies located in subtegumental cells (nucleated tegumental cell bodies). Labeled spherical bodies together with few elongated bodies occurred also in the anucleated tegumental syncytium (further reported as syncytium), however, less frequently then in other intravertebrate stages. The subtegumental cells were connected with syncytium *via* cytoplasmic bridges as reported for the bird schistosome of *T. szidati* [8] and human schistosomes [7,21]. Location of the above mentioned bodies in cercariae corresponds with the observations on *T. szidati* [8], except for the absence of these bodies in *T. regenti* cytoplasmic bridges in the present study.

In schistosomula, spherical bodies were the most reactive structures. Specific IgG recognized spherical bodies present in large amount in the syncytium and

cytoplasmic bridges, besides subtegumental cell bodies (Figure 2b); this indicates an increased transport of spherical bodies from subtegumental cells to the surface of developing schistosomula. Two different types of spherical bodies were present in the tegument of all the stages under study. Smaller bodies (200 nm in diameter) were more abundant than the bigger ones (300-350 nm in diameter). Both types of bodies were of similar electron density and immune mouse sera reacted with their content uniformly (Figure 2b-d). The antibody also bound to the elongated bodies present in the syncytium, but less frequently than to the spherical bodies. We suggest the analogy of observed elongated bodies with transport vesicles of human schistosomes, releasing their content after fusion with the tegument [22].

The surfaces of 3 and 5 days old schistosomula developed in duck, mouse or *in vitro* differ morphologically from the surface of 1 day old worms (spiniferous tegument of newly transformed schistosomula changes to a surface with pits, holes, and canals; [15]). Nevertheless, the antibody binding pattern of all schistosomula remained similar (Figure 2b-d). The antibody weakly bound also to circular and longitudinal muscles of all schistosomula (not illustrated).

In the case of adult worms, antibody binding was restricted mainly to the surface of the tegument and less frequently the antibody also recognized the spherical bodies located in the syncytium (not illustrated). Both reactions were of lower intensity compared to the other stages. It is possible that mouse sera were not able to recognize all of the antigens of adults, because schistosomula never mature in non-specific mammalian host. Nevertheless, comparison of the immunoreactivity of different stages showed a gradual decrease in the antigen abundance from cercariae to adult worms. Lower immunoreactivity therefore indicates that adult worms either did not express the same antigens as did the previous stages or they expressed them in lower amount.

It is known that the surface membrane of human schistosomes is renewed by means of abundant membraneous and elongated (discoid) bodies synthesized in subtegumental cells and transported to the surface [7,23]. Also the surface of *T. regenti* schistosomula undergoes intensive changes during development [15]; the spherical and elongated bodies recognized by IgG antibody in the present study may play the same role in delivery of molecules to the surface membrane of schistosomula. This can be deduced from the observation of immunoreactivity on schistosomulum surface membranes and their invaginations (tegumental pits; Figure 2d) and released parts of the tegumental membrane (not illustrated).

The antibody-binding structures detected in our study probably contain molecules exposed to the host immune system in particular stages/phases of migration; developmentally-regulated sets of such molecules are known for mammalian schistosomes [24,25] where special emphasis has been put on proteins playing a role in immune evasion and/or serving as anticipated vaccine or drug targets [26]. In this regard we can speculate about the nature and role of antigens detected in the present study.

Western blot analysis showed that several proteins of different molecular weight in the homogenate of *T. regenti* cercariae are specifically recognized by IgE and/or IgG from re-infected mice [5]. Subsequent mass spectrometry analysis identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) represented by the protein band of 34 kDa [27]. Molecular weight of 28 kDa of the other recognized protein band corresponds with the weight of glutathione S-transferase (GST) from human schistosomes. The exact ultrastructural localization of GAPDH and GST from human schistosome is unknown, but both enzymes are known to be associated with the tegument [28,29]. Based on these facts we hypothesize that the GAPDH and GST might also be expressed in *T. regenti* tegument.

Regarding the described localizations of *T. regenti* antigenic molecules, also highly immunogenic paramyosin must be kept in mind. The locations of non-filamentous form of paramyosin in sthe tegumental layer of *S. mansoni* and *S. japonicum* schistosomula and *S. mansoni* adults [30,31], as well as in postacetabular glands of *S. japonicum* cercariae [32] are in accordance with locations of structures detected in the present study. The stimulation of antibody production by antigens other than those suggested above, however, cannot be excluded for *T. regenti.*

In conclusion, our study on different life stages of *T. regenti* demonstrated changes in localization of antigenic molecules specifically recognized by IgG antibodies from sera of mice re-infected with *T. regenti*. These changes were caused by detachment of the most antigenic cercarial glycocalyx, and transport of highly antigenic spherical bodies from subtegumental cell bodies to the worm surface where release of their content takes place. Development of the worms was accompanied with progressive loss of immunoreactivity. Immunofluorescence as well as ultrastructural immunocytochemistry showed no differences in localization of antigens within schistosomula of the same age developed under different conditions. This implies that schistosomula in the non-specific host are likely able to produce tegument of similar antigenic features as in the specific definitive host.

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