Responses of the surface membrane and excretory system of *Schistosoma mansoni* to damage and to treatment with praziquantel and other biomolecules

F. A. OLIVEIRA[1], J. R. KUSEL[3], F. RIBEIRO[1] *and* P. M. Z. COELHO[2]*

[1] *Centro de Pesquisas René Rachou/Fiocruz, Belo-Horizonte, MG, Brasil*
[2] *Santa Casa de Misericórdia, Belo Horizonte, MG, Brasil*
[3] *IBLS, Glasgow University, Glasgow G12 8QQ, Scotland*

(*Received 5 August 2005; revised 27 August and 7 September 2005; accepted 7 September 2005; first published online 1 December 2005*)

SUMMARY

Damage to the surface membrane of adult *Schistosoma mansoni*, and the activity of the excretory system, as shown by resorufin fluorescence, was observed following treatment with praziquantel and incubation with other molecules. Praziquantel treatment induced damage to the surface membrane as measured by the use of a variety of fluorescent compounds. The excretory system of the male worm was inhibited immediately after praziquantel treatment, but fully recovered after culture for 2 h following removal of praziquantel. The excretory system of the female, observed to be minimally active in untreated worm pairs, was often greatly activated in paired females, as shown by intense resorufin labelling, after praziquantel treatment, and this continued during recovery of the male excretory system. In experiments with normal worm pairs, the female could be activated by inhibiting the metabolic rate of the pair by a cooling procedure. The effects on the excretory system of changes in culture conditions (such as changes in pH, concentrations of bacterial lipopolysaccharide, cytokines, reactive oxygen species, compounds which remove cholesterol, such as beta-methyl cyclodextrin, and damaging basic poly-L-lysine) were also assessed. It is concluded that the extensive excretory system of the adult worm is responsive to drug treatment and to certain changes in environmental conditions. Its activity seems to be strongly linked to the integrity of the surface membrane.

Key words: excretory system, *Schistosoma mansoni*, praziquantel, P-glycoprotein, cholesterol, beta-methyl cyclodextrin, poly-L-lysine, tumour necrosis factor-alpha.

INTRODUCTION

Recent work (Doenhoff *et al*. 2002; Davies *et al*. 2004) and information from the schistosome genome project (Verjovski-Almeida *et al*. 2003) has suggested that host and parasite live in a condition of mutual interdependence, in which each organism responds to signals from the other to maintain life and reproductive ability. The signals from the host are largely unknown but Verjovski-Almeida *et al*. (2003) suggested a multitude of possible hormones, cytokines and growth factors which may be recognized by receptors on the parasite. These authors also drew attention to molecules which may be secreted from the parasite and which may have an immunomodulatory function and may be involved in immune evasion (Verjovski-Almeida *et al*. 2004).

The interactions of the parasite with the host are thus extremely complex and the surface membrane is an interface between the 2 organisms. However, the integration of activities of 3 other organ systems are crucial to the life of the parasite, the nervous system, the gut and the excretory system.

The function of the excretory system is largely unknown but some aspects of its function are linked to the surface membrane in that *in vitro* damage to the membrane can result in retrograde uptake of macromolecules (Tan *et al*. 2003; Wippersteg *et al*. 2003).

The excretory system of the adult male and female *Schistosoma mansoni* has been well described morphologically (Wilson and Webster, 1974; Bogers *et al*. 1994) but its function under different conditions is not understood, although recent publications have indicated the presence in the excretory system of signalling molecules which may be of importance both in the response of the parasite to the host, and to the interactions between male and female worms (Finken *et al*. 1994; Mecozzi *et al*. 2000; Schechtman *et al*. 2001; Skelly and Shoemaker, 2001; Wippersteg *et al*. 2003). Sato, Kusel and Thornhill (2002, 2004) have implicated the P-glycoprotein and multi-resistant proteins in excretory activity and showed that resorufin was a P-glycoprotein substrate and the extent of its accumulation by the excretory system could be used as a measure of excretory activity.

* Corresponding author: Centro de Pesquisas René Rachou/Fiocruz, Belo Horizonte, MG, Brasil, Av. Augusto de Lima 1715, 30.190-002, Belo Horizonte, MG, Brasil. Tel: +55 31 3295 3566. Fax: +55 31 3295 3115. E-mail: coelhopm@cpqrr.fiocruz.br

Parasitology (2006), **132**, 321–330.
doi:10.1017/S0031182005009169 Printed in the United Kingdom

The P-glycoproteins are known to be activated or inhibited by a variety of environmental factors, such as stress, pH, cytokines, LPS and reactive oxygen species. Thus, with an important function of the P-glycoproteins in the excretory system, it is very likely that the excretory system of the schistosome is intimately, and perhaps in unexpected ways, involved in the host-parasite relationship. Thus, in this paper we will test the hypothesis that the excretory system is responsive to a variety of environmental stimuli, and in this way plays a vital role in the interaction of parasite and host.

MATERIALS AND METHODS

Reagents

Resorufin, Hoechst 33258, poly-L-lysine (molecular weight 84 kDa), rhodamine labelled *Glycine max* lectin and beta-methyl cyclodextrin were obtained from Sigma Chemical Co. St Louis, MO, USA. Praziquantel + enantiomer was a kind gift from Dr Achim Harder, LPS was a gift from Dr Ricardo Gazzinelli, TNF-alpha, a kind gift from Dr Juliana A. S. G. Estanislau, Hypnol (Merck, Sharp and Dohme,Inc.) was used as an anaesthetic for both mice and parasite. Texas Red Bovine Serum Albumin (Texas Red BSA) (Invitrogen, USA.).

Life-cycle of parasite and infection of animals

Swiss mice were infected with 50 cercariae of *Schistosoma mansoni* by the method of Smithers and Terry (1965). Cercariae were of the LE strain (Belo Horizonte) supplied by the Malacology Division of the CPqRR.

Incubations of adult worms and experiments in vitro

(1) *Labelling of excretory system of adult worms with resorufin and incubation with various effectors*. Adult worms were perfused by the method of Smithers and Terry (1965) using RPMI and added heparin as the perfusing fluid. Worms were washed in RPMI and 5% bovine foetal calf serum (FCS), and divided into Petri dishes (5 cm diameter) in 2·0 ml of RPMI medium and 5% bovine serum albumin. Additions of drugs or effector compounds were made to the Petri dish after the addition of the worms (5 pairs per Petri dish). After incubation of the worms in the Petri dishes, 25 μg in 10 μl of resorufin in methanol was added and incubated for a further 30 min to label the excretory system. Worms were then washed gently 5 times with 2·0 ml of RPMI and placed on a microscope slide within a Vaseline surround. To prevent movement of the worms when photography was required, a solution of Hypnol (MSD) (10% in RPMI) was added to the slide (50 μl RPMI, and an equal volume of Hypnol solution). The slides were then examined under the rhodamine filter of a Zeiss fluorescence microscope.

The following experiments were conducted with this labelling protocol: (a) resorufin excretion after 15 min praziquantel treatment, (b) resorufin excretion after 15 min praziquantel treatment and 2 h recovery in RPMI and 5% foetal calf serum (FCS); (c) 2 h after *in vivo* treatment with PZQ, mice were perfused to collect adult worms. Parasites were incubated for 30 min with resorufin. In some experiments the worms were labelled first and then treated with PZQ and other compounds.

(2) *Treatment with PZQ, LPS and beta-methyl cyclodextrin, hydrogen peroxide, tumour necrosis factor-alpha, and interferon gamma*. To enable us to quantify the immediate response to these compounds, adult worms were perfused by a modification of the method of Smithers and Terry (1965). We found that when worms left the mouse during perfusion they could be damaged if allowed to be collected on a plastic gauze, as is routine in this method. To avoid this damage the worms were perfused into a Petri dish containing 60 mg per ml bovine serum albumin. These worms were then transferred to another Petri dish with a plastic-tipped pipette and were incubated in medium (1 h at 37 °C) with the addition of 1·5 μg/ml resorufin and 10 μl of 1 mg/ml Hoechst 33258 to assess damage to worms (Lima *et al*. 1994). This lower concentration of resorufin yielded a less-pronounced labelling of tubules but allowed any activation or inhibition to be assessed more readily. Worms were washed very gently and placed singly within a Vaseline circle on a microscope slide, 4 rings per slide. This arrangement allowed us to observe the fluorescent labelling of the worms and to record any damage and activation of the worms before adding the effector molecules. Worms observed to be damaged were not used in the subsequent experiment. The reagent in growth medium was added to the worms in the Vaseline circles, but without the addition of a cover-slip or Hypnol. This obviated the development of anaerobiosis around the worms and allowed a more prolonged observation of the resorufin distribution (reduced resorufin is colourless). The activity of the excretory system was observed over a 30 min period and was scored by the method of Sato *et al*. (2002).

(3) *Recording and quantification of labelling of excretory system*. The tubules and branches of the excretory system were counted under the ×10 objective in the head, mid-body region and tail at different times after mounting the worms, and a system identical to that described in Sato *et al*. (2002) was used to evaluate the significance of the differences in excretory activity. In this method the fluorescent labelling of the excretory system of each male parasite was classified into one of the following groups (1)

https://doi.org/10.1017/S0031182005009169 Published online by Cambridge University Press

no tubules seen; (2) 1 tubule in tail, few branches, dye largely in nephridiopore; (3) 2 tubules in tail, few branches; (4) 2 tubules in tail, branches extensive in tail and a few in mid-region of body; (5) 2 strong main tubules; many tubules in centre of body; (6) 2 strong main tubules, branches in body and tail region; clear tubules and branches in head. Using the proportion of worms divided into each class, an index for each experiment was calculated as follows: (5 × proportion of '6' worms, +4 × proportion of '5', +3 × proportion of '4', +2 × proportion of '3' worms, +1 × proportion of '2' worms). Three experiments were carried out for each treatment and the mean and standard deviation of the indices calculated (Sato *et al*. 2002). For example, in an experiment with beta-methyl cyclodextrin, 5 male worms were scored: 5, 4, 4, 3, 2. The index is 3·6 thus: $(5 \times 1/5) + (4 \times 2/5) + (3 \times 1/5) + (2 \times 1/5)$.

The worms perfused for experimental investigation contain individuals with a wide range of excretory activities, and this is reflected in the high standard deviations of the results in Table 1. Photographic records were also taken with a digital camera (Cannon Rebel, model EOS 300D).

(4) *Detection of surface membrane damage by fluorescent probes*. (*a*) *Detection of damage*. Methods for detecting damage have been described by Lima *et al*. (1994). Briefly, after treatment with the relevant compound, and subsequent washing, worms were incubated for 1 h with 10 μl of Alexa fluor-phalloidin (50 μg/ml), rhodamine soy bean (*Glycine max*) lectin (5 μg/ml) and Hoechst 33258. Damage was assessed by fluorescence microscopy after washing the worms to remove the probes. (*b*) *Recovery from damage*. To investigate the repair of surface membranes damaged by praziquantel, adult worm pairs, treated with praziquantel for 15 min at 37 °C were divided into 2 portions, one portion was stained with the fluorescent probes and the other portion incubated for 2 h at 37 °C and then stained with the fluorescent probes. Incubated worms exhibited minimal staining.

Alternatively, individual worm pairs, whose damage was detected by fluorescent Hoechst 33258 patches on the body, and head were shown to be permeable in these regions to Texas-red BSA (10 μg/ml) incubated with the damaged parasite (Wippersteg *et al*. 2003). To detect repair of these regions worm pairs were incubated for 30 min subsequent to the 2 h at 37 °C with the Texas-red BSA. Repair was indicated by a lack of permeability to Texas-red BSA in the Hoechst 33258-stained region.

(5) *Treatment of adult worms with PZQ* in vitro *and* in vivo. *In vitro*. PZQ (isomer plus or commercial mixture in concentrations ranging from 0·1 to 5 μg/ml, were added to Petri dishes containing 5 pairs of adult worms. *In vivo*. Infected mice were treated through stomach tube with 400 mg per kg PZQ in distilled water. Mice were perfused 2 h after treatment so as to compare with the *in vitro* treatment.

Table 1. Worm treatments

(Worm pairs were labelled with 1 μg/ml resorufin for 1 h at 37 °C, washed thoroughly and treated as below. Worms were placed individually in the solution inside the Vaseline rings without a cover-slip. The values below are calculated from the scoring scheme of Sato *et al*. (2002), and are means and standard deviations of at least 3 independent experiments, with 5 worm pairs per experiment.)

Control	2·1 ± 0·38(6)
E. coli LPS (50 μg/ml)	2·1 ± 0·57(3)
Beta-methyl cyclodextrin (10 mg/ml)	3·5 ± 0·08(3)*
TNF-alpha (100 ng/ml)	2·1 ± 0·50(3) (male)
PZQ minus isomer (1 μg/ml)	0·16 ± 0·23(3)*
PZQ plus isomer (1 μg/ml)	0·36 ± 0·09(3)*
Poly-L-lysine (100 μg/ml)	3·4 ± 0·08(3)*

* Significantly different from control ($P < 0·001$).

(6) *Experiments to examine male-female interactions in excretory function*. Worm pairs, labelled with resorufin as above, were placed in RPMI medium with 5% FCS and cooled to 4 °C in the refrigerator and maintained there for 30 min. The worm pairs were then warmed to 37 °C, and observed on an open Vaseline rimmed slide (no cover-slip) under the fluorescence microscope.

(7) *Experiments to investigate the effects of worm muscle contraction on excretory activity*. One effect of praziquantel is to cause rapid muscle contraction. To examine whether muscle contraction alone is sufficient to disperse the resorufin from the tubules and branches, we compared the effects of praziquantel, (1 μg/ml), hydrogen peroxide (10 mM) (which affects intracellular membranes) and the xylocaine-based anaesthetic Rompun (10%), (Merck, Sharp and Dohme) (which affects neuromuscular function) on muscle contraction and resorufin distribution.

RESULTS

In order to test the hypothesis that the excretory system is responsive to a number of environmental, immunological and chemotherapeutic effectors, we describe below the effects of a number of treatments on the adult male and female worms. The effect of praziquantel, the drug of choice against schistosomiasis (Doenhoff *et al*. 2002) is described. This also revealed that the male worm influenced the activity of the female worm, and experiments linked to this observation follow. The effect of pH, various cytokines and LPS have been investigated due to their known effects on P-glycoproteins (Stavrovskaya, 2000). Finally, membrane perturbing agents were used, (poly-L-lysine, beta-methyl cyclodextrin),

https://doi.org/10.1017/S0031182005009169 Published online by Cambridge University Press

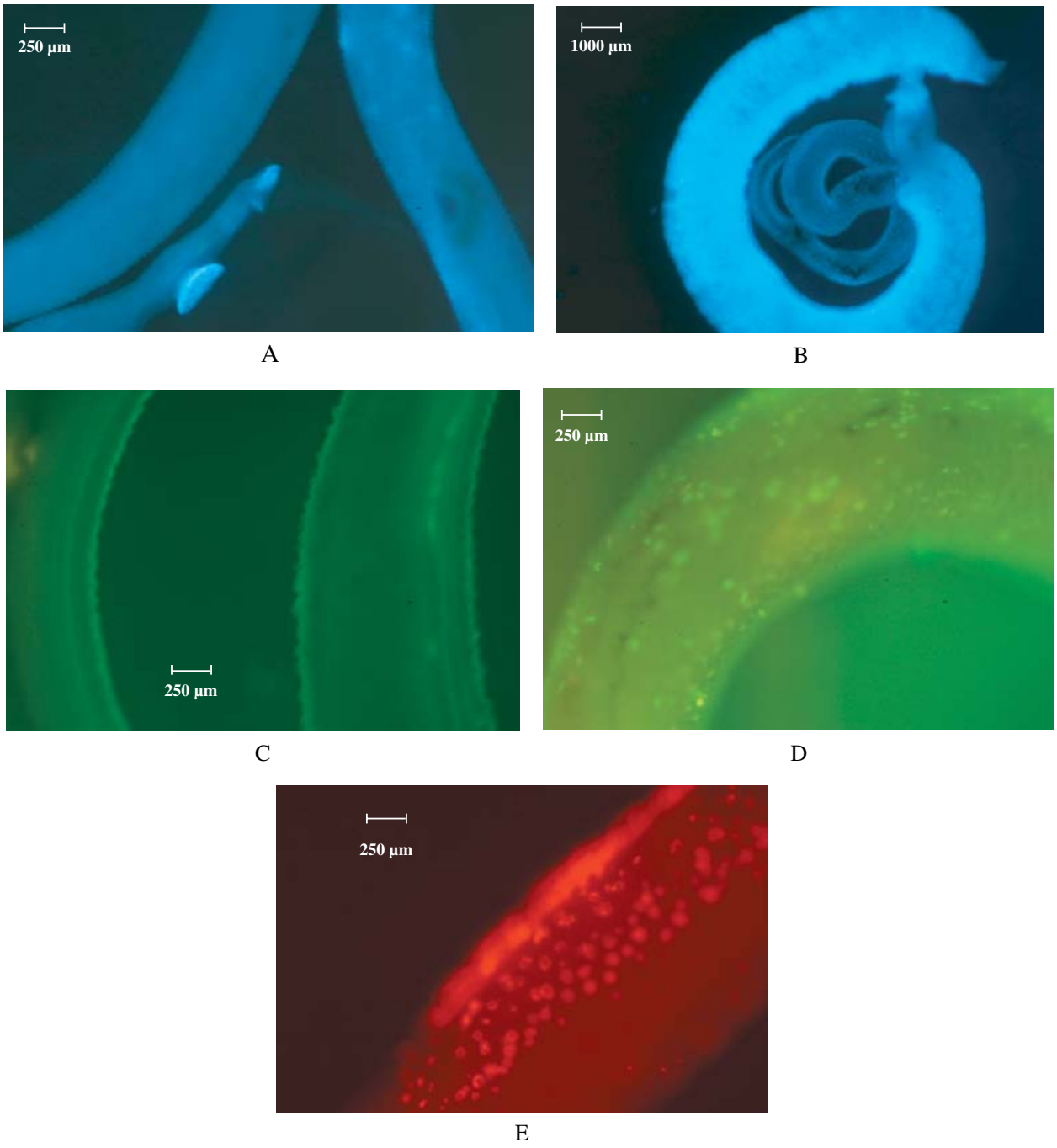

Fig. 1. Examples of the use of Hoechst 33258 (A) and (B), Alexa fluor-phalloidin (C) and (D) and rhodamine soybean lectin (E) in detecting damage to the adult male surface membrane by praziquantel after treatment *in vivo*. The lesions were repaired after 2 h in culture but not after drug treatment *in vivo*. (A) Represents the worms of the Hoechst 33258 control group, (B) PZQ treated, (C)Alexa fluor-phalloidin control group, (D) PZQ treated and (E) PZQ treated. The parasite treatment was performed with 400 mg/Kg PZQ *in vivo*. No control is shown for the rhodamine lectin since this compound did not bind to undamaged parasites.

since it has been found (Tan *et al*. 2003) that membrane damage can affect the excretory system.

Initial damage and recovery from damage

Surface damage as detected by staining with the Hoechst 33258, and the surface binding of fluorescent probes appeared after 15 min treatment with PZQ *in vitro* which was completely repaired after 2 h incubation. *In vivo* treatment showed similar amounts of damage with PZQ but after *in vivo* treatment with praziquantel, damage was not repaired (Fig. 1).

Repair of the surface membrane over a 2 h incubation period was shown by (a) loss of binding sites for rhodamine soy bean lectin (b) loss of binding of

https://doi.org/10.1017/S0031182005009169 Published online by Cambridge University Press

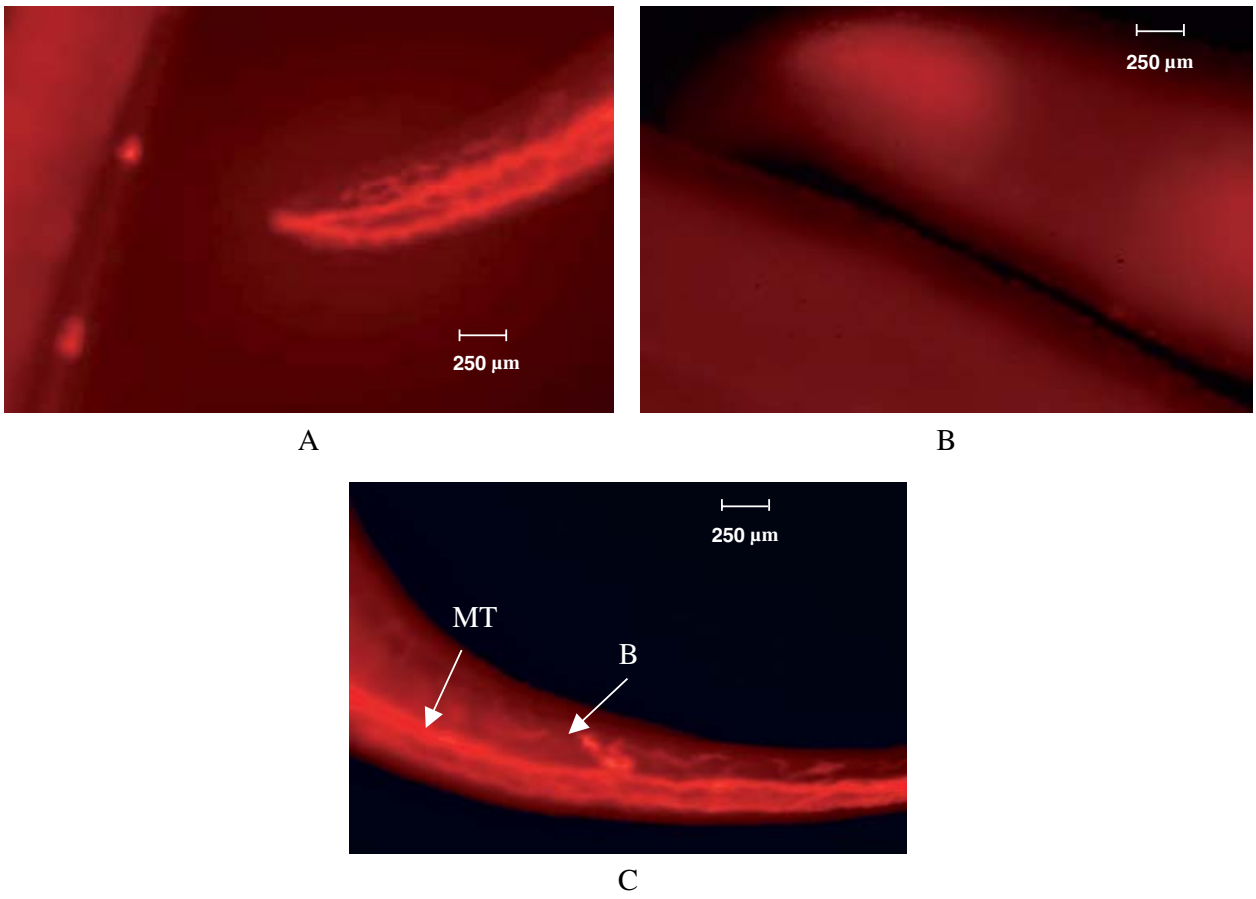

Fig. 2. Treatment of adult male worms labelled with resorufin (1 µg/ml) with praziquantel (2 µg/ml) *in vitro*. Complete dispersion of resorufin from the major excretory tubules is observed (B) when compared with the control untreated worms (A). Tubules in adult male worms recovering after incubation in culture medium after withdrawal praziquantel by extensive washing (C). Arrows show main tubules (MT) and branches (B).

Alexa fluor-phalloidin and (c) loss of staining of body regions by Hoechst 33258.

A method was employed using Texas-red BSA to reveal permeability to damaged Hoechst 33258-stained regions. Worms whose damage was indicated by Hoechst 33258 binding showed loss of permeability to Texas-red BSA after 2 h incubation, confirming the results from the other fluorescent probes that the membrane had repaired.

Effect of PZQ treatment in vitro *and* in vivo *as measured by resorufin entry and excretion from the cultured adult worm*

The labelling method using 25 µg/ml was used in the following experiments as in (1) above in the Materials and Methods section (Fig. 2A).

In vitro *treatment*. After 15 min treatment of adult worm pairs with PZQ very little excretory activity could be detected in either male or female worms (Fig. 2B). Two h after PZQ treatment and subsequent culture in the absence of praziquantel, the excretory system of the male worms had not only recovered, but had extensive labelling in tubules and fine branches, although this effect was variable from experiment to experiment (Fig. 2B). The effect of praziquantel added to male worms pre-labelled with resorufin was to disperse the resorufin in tubules and branches of parasites (Table 1; Fig. 2B). In contrast to the paired female after resorufin labelling, the isolated female can be clearly observed to have resorufin in the main tubules and in some branches of the excretory system (Fig. 4B). When 1 µg/ml praziquantel was added to isolated females, although considerable contraction and coiling took place, the resorufin remained confined to the excretory tubules. On recovery of the females from this treatment the tubules remained full of resorufin.

This effect of dispersal of resorufin from male excretory tubules was not due to muscle contraction alone, since when muscle contraction was induced by other methods (e.g. treatment with Rompun or hydrogen peroxide (10 mM), the dye was not dispersed from the tubules and branches. We interpret this to mean that there is an effect of praziquantel which leads to the dispersion of the dye additional to muscle contraction.

No significant difference could be detected between the isomers of praziquantel in their effects

https://doi.org/10.1017/S0031182005009169 Published online by Cambridge University Press

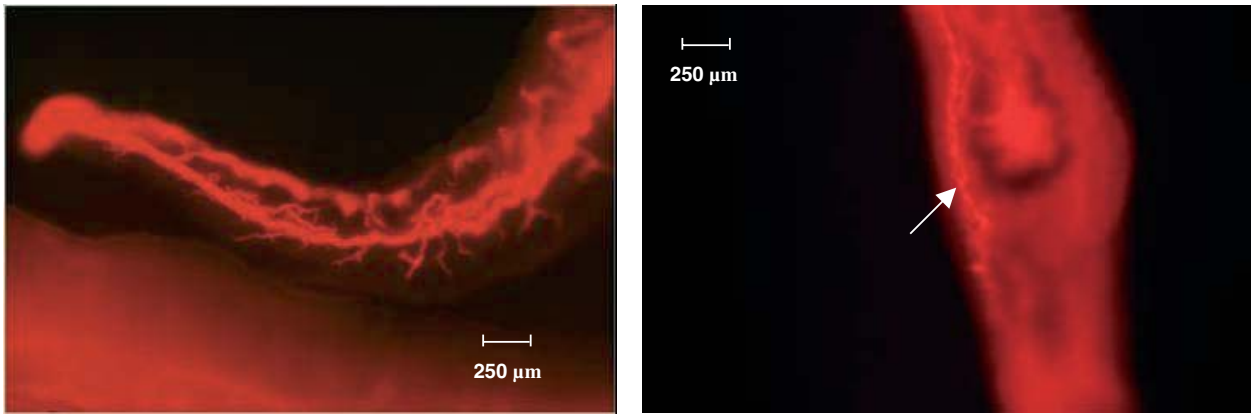

Fig. 3. Treatment of adult male worms labelled with resorufin (1 μg/ml) with beta-methyl cyclodextrin (10 mg/ml) *in vitro*. The tubules in the body are highly activated (A) and the head region shows some tubules (arrow) (B).

on the major excretory tubules in the range 1 to 5 μg/ml (Table 1).

Male influence on female excretory system

It was observed above that the paired female worm showed minimal activity of the excretory system, while females separated from the male showed enhanced activity with extensive labelling in tubules and branches (Fig. 4A, C). Damage to the male by praziquantel was observed to lead to the activation of the female excretory system of paired worms. This could be observed as soon as 30 min after treatment with between 0·1 and 5 μg/ml PZQ (Fig. 4B).

To study this activation of paired females further, worm pairs were labelled with resorufin (1·5 μg/ml) and, after extensive washing, were cooled to 4 °C for 30 min. On re-warming the labelled parasites, the females became activated although remaining paired. The males were living, excluded the Hoechst 33258, and were thus considered undamaged. The activation of the excretory system of the female thus appears to be related to slowing of the metabolism of the male worm.

Effect of external pH in vitro *on resorufin uptake and excretion*

The pH of the external medium was altered between pH 4·0 and pH 8·0 and worms were incubated for 2 h under these conditions. Worms were washed in the relevant low or high pH medium and labelled for 30 min in resorufin at pH 7·4. Very little change in the appearance of the excretory tubules was observed. This experiment was repeated using the 1·5 μg/ml labelling technique and the addition of medium of different pH values to worms on slides without cover-slips, and no effect of pH on the excretory tubules was seen.

Effect of beta-methyl cyclodextrin in vitro *on resorufin excretion*

The worms were labelled as described in the Materials and Methods section with 1·5 μg/ml resorufin. When beta-methyl cyclodextrin (10 mg/ml) was added to the microscope slide a substantial activation of the excretory system occurred in all males (Fig. 3A, B). This was seen in the extensive resorufin fluorescence in tubules and fine branches of the male. Very few female worms showed enhanced labelling.

Effect of Escherichia coli, lipopolysaccharide, hydrogen peroxide and tumour necrosis factor-alpha on the excretory system

No significant change in the extent of labelling of the excretory tubules was observed with any of these effectors in the assay described in Table 1.

The initial effect of adding LPS was to increase the number of small branches in the body of the male worms. After 30 min observation this effect disappeared and the whole excretory system became similar to controls. This increase approached but did not reach statistical significance.

Hydrogen peroxide (10 mM) caused contraction of the worms and no activation was observed. When 0·5 mM hydrogen peroxide was used branches were observed in the body surface of the worm, but the numbers of worms carrying these was not significantly greater than in the controls. Like hydrogen peroxide, TNF-alpha (5 ng/ml) induced branches also but the results lacked statistical significance.

Effect of the basic protein, poly-L-lysine (molecular weight 84 kDa)

Poly-L-lysine can be used as a model for eosinophil major basic protein (Tan *et al.* 2003). A range

https://doi.org/10.1017/S0031182005009169 Published online by Cambridge University Press

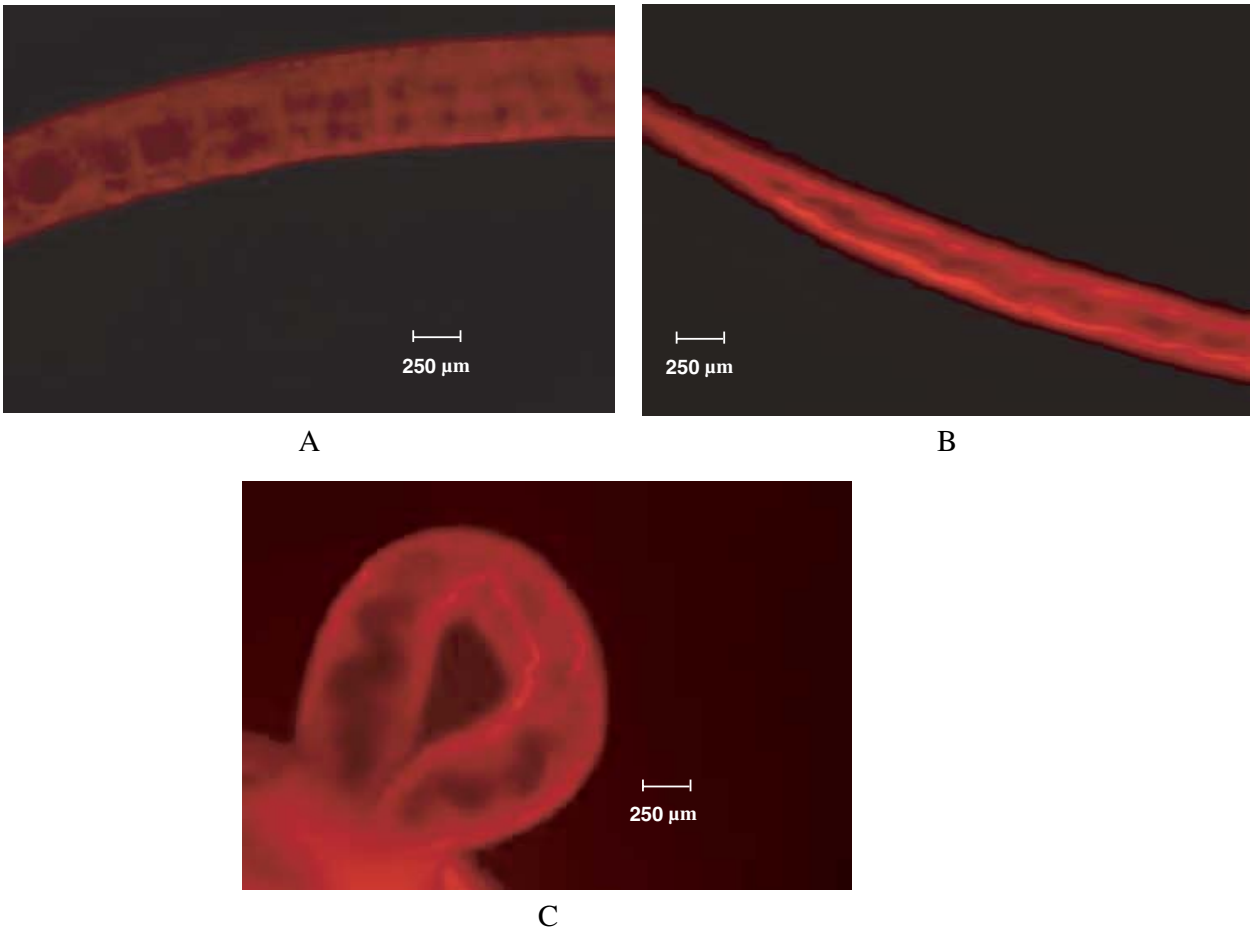

Fig. 4. Adult worm pairs and isolated female worms were labelled with resorufin (10 μg/ml). The female worm, when paired shows little labelling of main tubules (A). When separated from the male considerable activity is seen (B). After praziquantel treatment *in vitro* or *in vivo* the paired female worm becomes activated (C). No dispersion of resorufin from isolated female tubules occurs after praziquantel treatment.

of concentrations induced activation of the excretory system with increased resorufin fluorescence in the numerous branches and main tubules of the excretory system of male worms (Table 1).

DISCUSSION

The morphology of the excretory system of platyhelminths (Wilson and Webster, 1974) has been studied for many years but there has been little experimental evidence for its function in any organism, and its role in parasitic forms is uncertain (Mecozzi *et al*. 2000). It has been shown to secrete small molecules, ions and even immunogenic proteins and enzymes, and a number of possible functions of the system have been suggested. The excretory products might regulate the external environment of the parasite, have an immunomodulatory function (Mountford and Trottein, 2004), signal to other members of the population, or be involved in quorum sensing. As a first step in understanding the function of the excretory system in schistosomes it has been the hypothesis in this paper that the excretory system can respond to changes in the external environment of the adult parasite. It can be seen from the results that the activity of the excretory system is very responsive to praziquantel, removal of membrane cholesterol and membrane damage by cationic polypeptides. It is a very interesting finding also that the excretory activity of the female is inhibited when paired with the male, unless the male is damaged in some way, by drugs, surface membrane damage or a decrease in metabolism by cooling.

It would be most interesting to study the signalling pathways which allow the excretory system to sense the changes in the external environment. Mecozzi *et al*. 2000, have drawn attention to the likely role of ion fluxes in affecting flame cell activity, and the P-glycoproteins responsible for pumping the substrate resorufin (Sato *et al*. 2002, 2004) are likely to be affected by cytokines, TNF-alpha, drugs, reactive oxygen species, and stress (Stavrovskaya, 2000). One signalling pathway in which P-glycoproteins are activated is via their phosphorylation (Idriss *et al*. 2000), and further work is needed to elucidate whether this is case. Our results with beta-methyl cyclodextrin, a compound that removes cholesterol

https://doi.org/10.1017/S0031182005009169 Published online by Cambridge University Press

from membranes (El Ridi *et al*. 2004) suggest that the excretory system may also be involved in sensing cholesterol in the membrane. This will be discussed further below.

The activity of the excretory system *in vitro* has been described by Sato *et al*. (2002, 2004). We describe in this paper the effect of praziquantel and other effectors on the surface membrane and excretory system of adult male and female worms.

The basic polypeptide, poly-L-lysine caused activation of the excretory system at a range of concentrations. We had also noticed that any physical damage during perfusion also activated the system. We modified the perfusion technique to minimize damage during perfusion.

The treatment both *in vitro* and *in vivo* with PZQ induced damage to the tegument of the male worm, as detected by the fluorescent probes. Surface damage was detected by increased permeability to the fluorescent Hoechst 33258 in both male and female worms.

PZQ, when added to the worm pairs completely inhibited the excretory system in its uptake of resorufin, and caused the dispersal of the dye from pre-labelled male worms. This effect was shown to be a feature of PZQ action on the tubules and branches of the excretory system, and not just an effect of the muscle contraction alone. The effect of PZQ may be directly on the tubule cell membrane, on the P-glycoproteins, or an indirect effect due to diminished ATP or increase in cell calcium concentration. When PZQ-treated worms were washed and cultured for 2 h, as described above, the surface and the excretory system made a remarkable recovery, many worms showing activity greater than in any of the control worms. Paired female worms often showed activity under these conditions and showed enhanced excretion of resorufin. Since under normal culture conditions isolated females show uptake and excretion of resorufin, while paired females show much less activity, it is possible that the undamaged male exercises an inhibitory effect on the P-glycoprotein in the female protonephridia. This may explain why Sato *et al*. (2002) found that the female was often activated by cyclosporin A and other P-glycoprotein inhibitors, when the male was very evidently inhibited. The influence of the male on the female may be mediated by hydrophobic compounds (Shaw, 1987) such as steroids, or other lipids (Borst, Zelcer and van Helvoort, 2000) which can be potent P-glycoprotein substrates.

When we looked at other environmental effects *in vitro* on the excretory system, we found that the external pH has no effect, showing that very efficient intracellular buffering systems are in place (Pax and Bennett, 1990). No consistent prolonged effect of reactive oxygen species (hydrogen peroxide), bacterial lipopolysaccharide, tumour necrosis factor-alpha, or interferon-gamma was observed. P-glycoprotein of mammalian cells has been reported to respond to cytokines (Sukai and Piquette-Miller, 2000).

A dramatic and highly consistent increase in activity of the excretory system with extensive branching was observed when labelled worms came in contact with beta-methyl cyclodextrin. This compound has been used to deplete cholesterol from membrane rafts (Heino *et al*. 2000). The removal of cholesterol and other lipids from the surface membrane by beta-methyl cyclodextrin has been shown by El Ridi *et al*. (2004) to expose surface antigens. In our work, beta-methyl cyclodextrin seems to give a rapid and potent signal to the excretory P-glycoproteins. This may be a signal to the parasite to increase lipid transport in the entire worm. Movement of cholesterol from the surface to internal structures has been demonstrated (Moffat and Kusel, 1992). Reverse transport may also occur (Heino *et al*. 2000; Lange *et al*. 1999). There is evidence that P-glycoprotein activity can be sensitive to cholesterol concentrations (Rothnie *et al*. 2001) and is involved in cholesterol transport (Metherall, Li and Waugh, 1996). Cholesterol-sensing proteins other than P-glycoproteins may also be involved in these processes.

This suggests that the proteins responsible for the pumping of resorufin in the male worm (we propose these are P glycoproteins (Sato *et al*. 2002), but there may be others) are sensitive to cholesterol concentrations in the surface membrane or in the medium. The domain or raft structure of the surface (Foley *et al*. 1986) may be important in signalling these changes in cholesterol concentration. It has been shown (Rumjanek and McLaren, 1981) that depletion of cholesterol from schistosomula renders them more susceptible to surface membrane damage by eosinophils. Thus, the response of the excretory system to beta-methyl cyclodextrin may represent a general activation response when certain forms of surface damage are detected by the parasite. The activation of the excretory system seen after treatment of labelled worms with poly-L-lysine (an experimental model for eosinophil major basic protein) (Tan *et al*. 2003) is consistent with this hypothesis, and may involve increased ion permeability (Kleine, Gleich and Lewis, 1998).

In conclusion, our results show that both the membrane and excretory system can recover from damage by praziquantel. The excretory system of the paired female can be activated by this treatment as a result of effects on the male worm. The excretory system of the male may be involved in cholesterol transport and act as a sensor for cholesterol concentration in the surface membrane, or in the external medium, and appears to be activated whenever the integrity of the surface membrane is compromised. The excretory system thus appears to be highly responsive to some host signals, and may play a role

in both the regulation of worm metabolism and its response to drugs and the immune system.

We would like to thank Dr Ricardo Gazzinelli for the gift of *E.coli* lipopolysacharide, and Dr Juliana A. S. G. Estanislau for the gift of tumour necrosis factor-alpha. We are very grateful for CAPES for funding the Fellowship of J. R. K. We are also grateful for Centro de Pesquisas René Rachou/FIOCRUZ for providing Fabricia Alvisi de Oliveira a Master Degree Fellowship.

REFERENCES

Bogers, J. J., Nibbeling, H. A., van Marck, E. A. and Deelder, A. M. (1994). Immunofluorescent visualisation of the excretory and gut system of *Schistosoma mansoni* by confocal laser scanning microscopy. *American Journal of Tropical Medicine and Hygiene* **50**, 612–619.

Borst, P., Zelcer, N. and van Helvoort, A. (2000). ABC transporters in lipid transport. *Biochimica et Biophysica Acta* **1486**, 128–144.

Cioli, D., Pica-Mattoccia, L. and Archer, S. (1993). Drug resistance in schistosomes. *Parasitology Today* **9**, 162–166.

Davies, S. J., Lim, K. C., Blank, R. B., Kim, J. H., Lucas, K. D., Hernandez, D. C., Sedgwick, J. D. and McKerrow, J. H. (2004). Involvement of TNF in limiting liver pathology and promoting parasite survival during schistosome infection. *International Journal for Parasitology* **34**, 27–36.

Doenhoff, M. J., Kusel, J. R., Coles, C. G. and Cioli, D. (2002). Resistance of *Schistosoma mansoni* to praziquantel: is there are problem? *Transactions of the Royal Society of Tropical Medicine and Hygiene* **96**, 465–469.

Doenhoff, M. J., Stanley, R. G., Griffiths, K. and Jackson, C. L. (2002). An anti-atherogenic effect of *Schistosoma mansoni* infections in mice associated with a parasite induced lowering of blood total cholesterol. *Parasitology* **125**, 415–421.

El Ridi, R., Tallima, H., Mohamed, S. S. and Montash, M. (2004). Depletion of *Schistosoma mansoni* lung stage schistomula cholesterol by methyl-beta cyclodextrin dramatically increases specific antibody binding to surface antigens. *Journal of Parasitology* **90**, 727–732.

Finken, M., Sobek, A., Symmons, P. and Kunz, W. (1994). Characterisation of the complete protein disulfide isomerase gene of *Schistosoma mansoni* and identification of the tissues of its expression. *Molecular and Biochemical Parasitology* **64**, 135–144.

Foley, M., MacGregor, A. M., Kusel, J. R., Garland, P. B., Downie, T. and More, I. (1986). The lateral diffusion of lipid probes in the surface membrane of *Schistosoma mansoni*. *Journal of Cell Biology* **103**, 807–818.

Heino, S., Lusa, S., Somerharju, P., Ehnholm, C., Olkkonen, V. M. and Ikonen, E. (2000). Dissecting the role of the Golgi complex and lipid rafts in biosynthetic transport of cholesterol to the cell surface. *Proceedings of the National Academy of Sciences, USA* **97**, 8375–8380.

Idriss, H. T., Hannun, Y. A., Boelpaep, E. and Bawpappa, S. (2000). Regulation of volume activated chloride channels by P-glycoprotein: phosphorylation has the final say. *Journal of Physiology* **524**, 624–636.

Jones, J. T. and Kusel, J. R. (1989). Intra-specific variation in *Schistosoma mansoni*. *Parasitology Today* **5**, 37–39.

Kleine, T. J., Gleich, G. J. and Lewis, S. A. (1998). Eosinophil major basic protein increases permeability in mammalian urinary bladder epithelium. *American Journal of Physiology: Cellular Physiology* **275**, C93–C103.

Lange, Y., Ye, J., Rigney, M. and Steck, T. L. (1999). Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol. *Journal of Lipid Research* **40**, 2264–2270.

Lima, S. F., Vieira, L. Q., Harder, A. and Kusel, J. R. (1994). Altered behaviour of carbohydrate bound molecules and lipids in areas of the tegument of adult *Schistosoma mansoni* damaged by praziquantel. *Parasitology* **109**, 469–477.

Mecozzi, B., Rossi, A., Lazzaretti, P., Kady, M., Kaiser, S., Valle, C., Cioli, D. and Klinkert, M. Q. (2000). Molecular cloning of *Schistosoma mansoni* calcineurin subunits and immunolocalisation to the excretory system. *Molecular and Biochemical Parasitology* **110**, 333–343.

Metherall, J. E., Li, L. and Waugh, K. (1996). Role of multidrug resistance glycoproteins in cholesterol biosynthesis. *Journal of Biological Chemistry* **271**, 2634–2640.

Moffat, D. and Kusel, J. R. (1992). Fluorescent lipid uptake and transport in adult *Schistosoma mansoni*. *Parasitology* **105**, 81–89.

Mountford, A. P. and Trottein, F. (2004). Schistosomes in the skin: a balance between immune priming and regulation. *Trends in Parasitology* **20**, 221–226.

Pax, R. A. and Bennett, J. L. (1990). Studies on intrategumental pH and its regulation in adult male *Schistosoma mansoni*. *Parasitology* **101**, 219–226.

Rothnie, A., Theron, D., Soceneatu, L., Martin, C., Traikia, M., Berridge, G., Higgins, C., Phillipe, F. and Callaghan, R. (2001). The importance of cholesterol in the maintenance of P-glycoprotein activity, and its membrane perturbing influence. *European Biophysics Journal* **30**, 430–442.

Rumjanek, F. D. and McLaren, D. J. (1981). *Schistosoma mansoni*: modulation of schistosomular lipid composition by serum. *Molecular and Biochemical Parasitology* **3**, 239–252.

Sato, H., Kusel, J. R. and Thornhill, J. A. (2002). Functional visualisation of the excretory system of adult *Schistosoma mansoni* by the fluorescent marker resorufin. *Parasitology* **125**, 527–535.

Sato, H., Kusel, J. R. and Thornhill, J. A. (2004). Excretion of fluorescent substrates of mammalian multidrug resistance associated protein (MRP) in the *Schistosoma mansoni* excretory system. *Parasitology* **128**, 43–52.

Schechtman, D., Winnen, R., Tarrab-Hazdai, R., Ram, D., Schinder, V., Grevelding, C. G., Kunz, W. and Arnon, R. (2001). Expression and immunolocalization of the 14-3-3 protein of *Schistosoma mansoni*. *Parasitology* **123**, 573–582.

Skelly, P. J. and Shoemaker, C. B. (2001). *Schistosoma mansoni* proteases Sm 31(cathepsin b) and Sm32

(legumain) are expressed in the caecum and protonephridia of cercariae. *Journal of Parasitology* **87**, 1218–1221.

Shaw, M. K. (1987). *Schistosoma mansoni*: vitelline gland development in females from single sex infections. *Journal of Helminthology* **61**, 253–259.

Smithers, S. R. and Terry, R. J. (1965). The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and recovery of adult worms. *Parasitology* **55**, 695–700.

Stavroyskaya, A. A. (2000). Cellular mechanisms of multidrug resistance of tumour cells. *Biochemistry* **65**, 95–106.

Sukai, M. and Piquette-Miller, M. (2000). Regulation of multidrug resistance genes by stress signals. *Journal of Pharmacological Pharmaceutics* **3**, 268–280.

Tan, H. H., Thornhill, J. A., Al-Adhami, B. H., Akhkha, A. and Kusel, J. R. (2003). A study of the effect of surface damage on the uptake of Texas Red BSA by schistosomula of *Schistosoma mansoni*. *Parasitology* **126**, 235–240.

Verjovski-Almeida, S., DeMarco, R., Martins, E. A. L., Guimaraes, P. E. M., Ojopi, E. P. B., Paquola, A. C. M., Piazza, J. P., Nishyama M. Y., Jr, Kitajima, J. P., Adamson, R. E., Ashton, P. D., Bonaldo, M. F., Coulson, P. S., Dillon, G. P., Farias, L. P., Gregorio, S. P., Ho, P. L., Leite, R. A., Malaquias, L. C. C., Marques, R. C. P., Miyasoto, P. A., Nascimento, A. L. T. O., Ohlweiler, F. P., Reis, E. M., Ribeiro, M. A., Sa, R. G., Stukart, G. C., Soares, M. B., Gargioni, C., Kawano, T., Rodrigues, V., Madeira, A. M. B. N., Wilson, R. A., Menck, C. F. M., Setubal, J. C., Leite, L. C. C. and Dias-Neto, E. (2003). Transcriptome analysis of the acoelomate human parasite *Schistosoma mansoni*. *Nature Genetics* **35**, 148–157.

Verjovski-Almeida, S., Leite, L., Dias-Neto, E., Menck, C. F. M. and Wilson, R. A. (2004). Schistosome transcriptome: insights and perspectives for functional genomics. *Trends in Parasitology* **20**, 304–308.

Wilson, R. A. and Webster, L. A. (1974). Protonephridia. *Biological Reviews* **49**, 127–160.

Wippersteg, V., Ribeiro, F., Liedke, S., Kusel, J. R. and Grevelding, C. G. (2003). The uptake of Texas Red BSA in the excretory system of schistosomes and its colocalisation with ER60 promoter induced GFP in transiently transformed adult males. *International Journal for Parasitology* **33**, 1139–1143.

https://doi.org/10.1017/S0031182005009169 Published online by Cambridge University Press