Journal of Helminthology (2002) **76,** 327–333 DOI: 10.1079/JOH2002135

Adult worm tegumental damage and egg-granulomas in praziquantel-resistant and -susceptible *Schistosoma mansoni* treated *in vivo*

Y.-S. Liang[1,2], G.C. Coles[2]*, J.-R. Dai[1], Y.-C. Zhu[1] and M.J. Doenhoff[3]

[1]Jiangsu Institute of Parasitic Diseases, Meiyuan, Wuxi, Jiangsu 214064, People's Republic of China: [2]Department of Clinical Veterinary Science, University of Bristol, Langford House, Langford, Bristol, BS40 5DU, UK: [3]School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, UK

Abstract

The effect of treatment with praziquantel (PZQ) on the tegument of adult *Schistosoma mansoni* worms and on liver egg-granulomas has been examined in mice infected with PZQ-resistant and -susceptible parasite isolates. Two PZQ-resistant *S. mansoni* isolates, one selected by passage in the laboratory under drug pressure and one from Senegal established from eggs excreted by an uncured patient, were compared with PZQ-susceptible control isolates. Scanning electron microscopic observations on the tegument of *Schistosoma* adult worms treated *in vivo* with PZQ showed that more severe damage was inflicted by PZQ on susceptible worms than on drug-resistant worms. Observations on the pathology of *Schistosoma* egg-granulomas in the liver of infected mice after treatment with PZQ indicated that eggs from susceptible control isolates were more sensitive to PZQ than those from drug-resistant isolates.

Introduction

Praziquantel (PZQ) is now the drug of choice for the treatment of human schistosomiasis (World Health Organization, 1993) and it is also increasingly being used for morbidity control. However, widespread use of a drug to kill parasites raises the risk of selecting resistance to that drug in the target organism. Laboratory studies (Fallon & Doenhoff, 1994) and results from field isolates from both Egypt (Ismail *et al.*, 1996) and Senegal (Gryseels *et al.*, 1994; Fallon *et al.*, 1995; Stelma *et al.*, 1995) have shown that PZQ-resistance can occur in some isolates of *Schistosoma mansoni*. Praziquantel resistance can be tested for by treating infected mice or by *in vitro* tests, e.g. by observing contractility of adult worms or tegument damage (Sobhon & Upatham, 1990; Ismail *et al.*, 1999). A recent report described differences in cure rates between susceptible and laboratory-selected populations (Liang *et al.*, 2001). The present study extends these observations to an examination of the effects of *in vivo* drug treatment on the tegument of adult worms and on the resolution of egg-granulomas in host tissue.

*Fax: +44 (0) 117 928 9505
E-mail: gerald.c.coles@bristol.ac.uk

Materials and methods

Parasite and snail isolates

Four PZQ-susceptible isolates of *Schistosoma mansoni* were used. These were a 'gene-pooled' isolate which was

originally prepared by mixing equal numbers of cercariae of four susceptible isolates from different geographic areas and passaging the hybrid mixture in mice and snails as described by Fallon & Doenhoff (1994); two isolates from Kenya, one of which has been laboratory-passaged since 1982 (Kenya-lab) and one which was obtained in 1994 (Kenya-field); both having been described and studied previously (Fallon *et al.*, 1997); and one isolate from Egypt (Egyptian), the origin of which was outlined in Dunne *et al.* (1984).

Two PZQ-resistant isolates were used. One had been selectively bred for PZQ resistance by subjecting successive mouse passages of the 'gene-pooled' isolate (Lab-selected resistant) referred to above to sub-curative treatments of PZQ (Fallon & Doenhoff, 1994). The second was a Senegalese isolate (Senegalese-2) established from eggs excreted by an uncured patient treated with PZQ. The drug-sensitivity/resistance profiles of all the above isolates have recently been evaluated by treating mice with mature infections with $3 \times 200\,mg\,kg^{-1}$ PZQ. The two resistant isolates were less responsive to PZQ than the four susceptible ones (64–74% reduction in worm burden compared to 92–100%; Liang *et al.*, 2001).

The methods of routine maintenance of the parasite isolates were as described by Liang *et al.* (2001). Albino Puerto Rican *Biomphalaria glabrata* snails were used as the intermediate hosts to maintain the parasites.

Electron microscopy

Two isolates of *S. mansoni*, the 'gene-pooled' susceptible and the lab-selected resistant isolate, were used in these experiments. Each isolate was used to infect a group of 18 mice with 200 cercariae by Standen's modified paddling

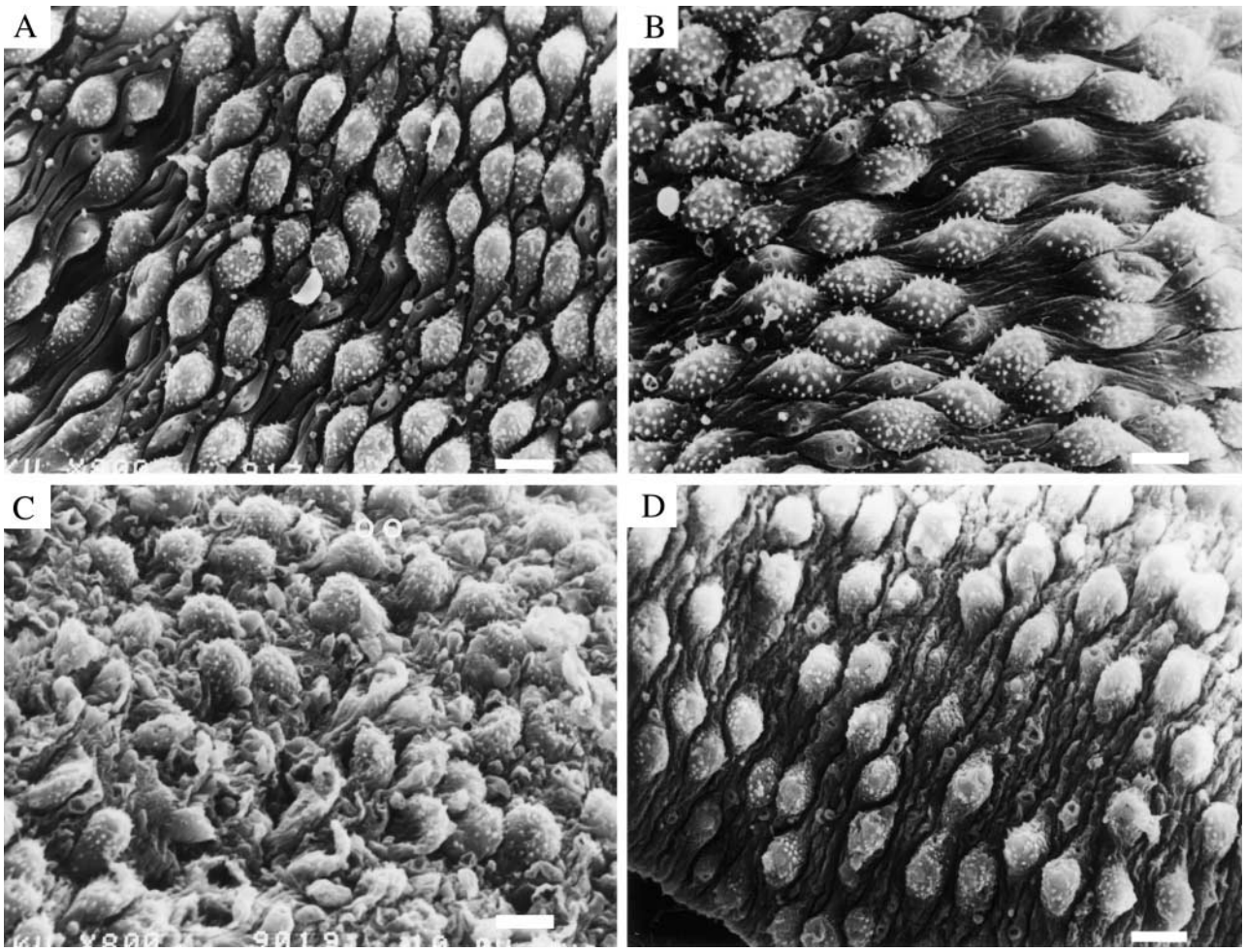

Fig. 1. Scanning electron micrographs showing the tegumental surface of adult male *Schistosoma mansoni* worms. A. Worm of the 'gene-pooled' PZQ-susceptible isolate collected 10 min after treatment of mice with $300\,mg\,kg^{-1}$ PZQ, showing numerous small spherical discrete blebs on the tegumental surface. B. Worm of the lab-selected PZQ-resistant isolate collected 10 min after treatment of mice with $300\,mg\,kg^{-1}$ PZQ, showing only a few blebs on the tegumental surface. C. Worm of the susceptible isolate 30 min after drug-treatment showing that blebs become more numerous, larger and more widespread. Some blebs were ruptured to form tegumental lesions. D. Worm of the lab-selected resistant isolate 30 min after drug treatment, showing less numerous blebs and less pronounced lesions on the tegument. Bar = 10 μm.

https://doi.org/10.1079/JOH2002135 Published online by Cambridge University Press

method (Standen, 1949). On day 57 after infection, the mice were treated with a single sub-curative dose of micronized PZQ (300 mg kg^{-1}) by oral gavage in an aqueous suspension. At 10 and 30 min and 1, 12, and 24 h after dosing, three mice from each of the groups infected with the two isolates were killed and adult worms recovered immediately by portal perfusion (Radke *et al.*, 1961; Smithers & Terry, 1965).

Worms collected in the perfusates were washed several times with saline and fixed for 24–48 h in 5% glutaraldehyde at 4°C. Post-fixation (2% OsO4) took place for 2 h. The fixed and osmium-treated samples were dehydrated in graded acetone, transferred to liquid CO_2, and critical-point-dried. Dried samples were mounted on metal stubs, sputtered with gold in a SEM E 5100 coating unit, and observed using a S-520 scanning electron microscope (HITACHI, Japan). Four female and four male worms were examined for each isolate at each time point.

Pathology of egg-granulomas

Five isolates of *S. mansoni* were used. These were two PZQ-resistant isolates (i.e. the lab-selected resistant and the Senegalese-2 isolates) and three PZQ-susceptible isolates (the Egyptian, Kenya-field and Kenya-lab isolates). Each isolate was used to infect 20 mice (200 cercariae per mouse) and the mice were divided into two groups, a control group and a treated group. On each of days 58 to 60 post-infection 200 mg kg^{-1} PZQ were administered to the treated groups. All mice in both treated and control groups were killed on day 35 post-treatment with PZQ and livers were collected for pathological examination.

Liver samples were fixed in absolute alcohol for 24 h, then in 90%, 80% and 70% alcohol for 24 h respectively, embedded in paraffin, cut in sections at 7 μm and stained with haematoxylin and eosin for microscopic observation.

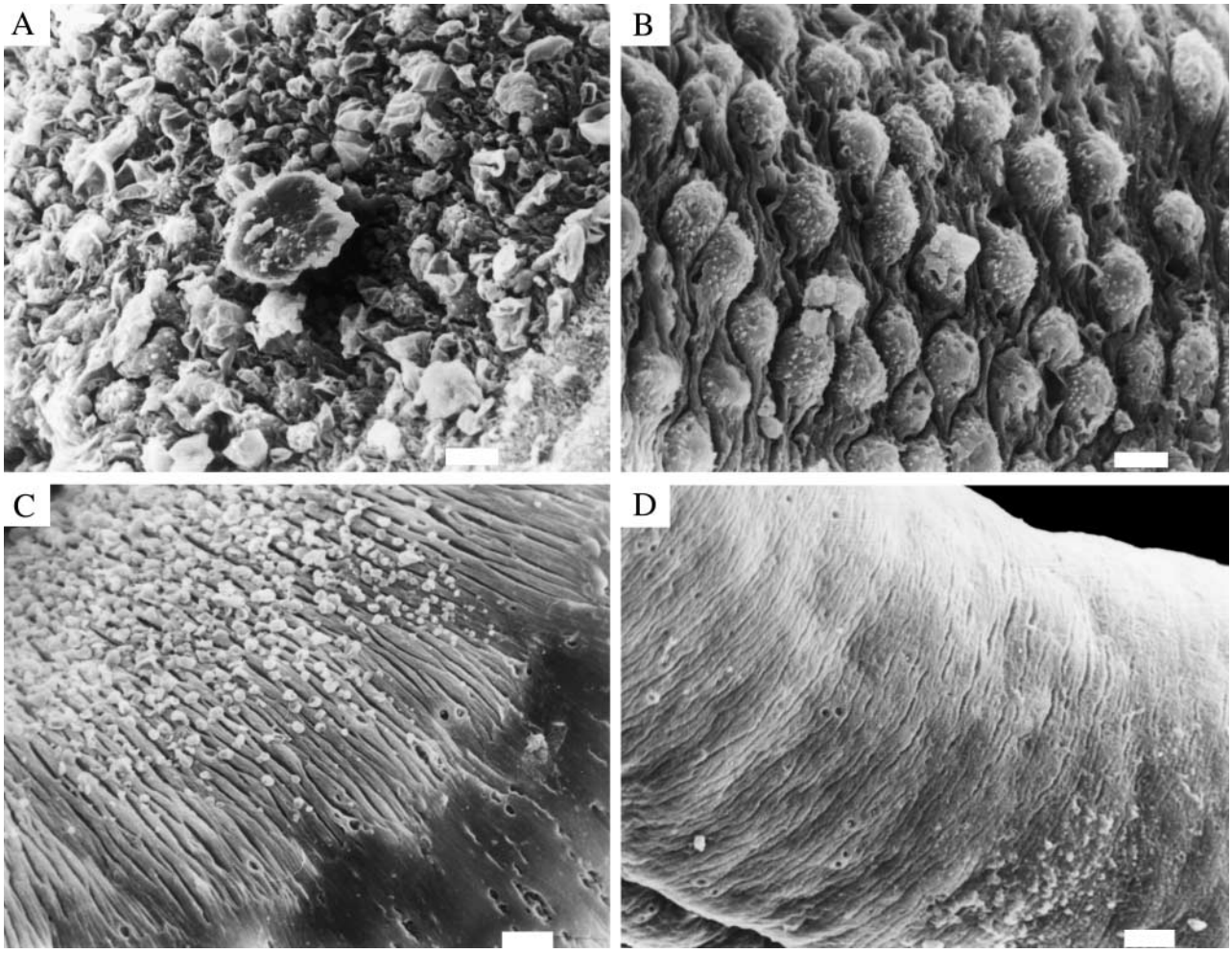

Fig. 2. Scanning electron micrographs showing the tegumental surface of adult *Schistosoma mansoni* worms. A. Male worm of the susceptible isolate 12 h after treatment, showing ruptured blebs throughout the surface, no normal tubercles were present on the tegumental surface. B. Male worm of the lab-selected resistant isolate 12 h after treatment, showing some blebs and disrupted blebs on the tegumental surface. C. Female worm of the susceptible isolate 12 h after treatment showing damaged areas and small blebs on the tegumental surface. D. Female worm of the lab-selected resistant isolate 12 h after treatment, showing only a few small blebs on a limited area of the tegumental surface. Bar = 10 μm.

https://doi.org/10.1079/JOH2002135 Published online by Cambridge University Press

The areas and circumferences of egg-granulomas surrounding single eggs containing a mature miracidium were measured using a VIDS-III Analytical Measuring System (AMS, Cambridge, UK). For each isolate, 40 egg-granulomas were measured, 20 from the control group and 20 from the treated group.

Statistical analysis

Analysis of variance and Dunnet's *t*-test were used to test for significance of difference between groups. *P* values <0.05 were considered significant.

Results

Electron microscopy

The damage induced by PZQ to the tegument of *S. mansoni* consisted mainly of the formation of bleb-like structures protruding out from the surface. Most of these blebs were thin-walled. The blebs were not present on the surface of untreated worms.

Ten minutes after dosing infected mice with $300\,mg\,kg^{-1}$ PZQ, male worms showed a strong contraction compared to untreated worms. On the tegument of susceptible male worms numerous small spherical discrete blebs were present, mostly in the area between spine-bearing tubercles on the dorsal surface (fig. 1A). Only a few blebs were found on the surface of male worms in the drug-resistant isolate (fig. 1B).

Thirty minutes later, more than 90% of male worms in the susceptible isolate were showing tegumental alterations, and the surface blebs became more numerous, larger and more widespread on the tubercular surface and the areas between tubercles. Some blebs ruptured to form tegumental lesion spots (fig. 1C). The blebs and lesions were less numerous and less pronounced on male worms of the drug-resistant isolate than on the susceptible isolate (fig. 1D).

One hour after dosing, numerous blebs had formed across the surface, and some blebs and tubercles had ruptured to form lesions on susceptible worms, while most of the remaining tubercles were in a swollen state. A

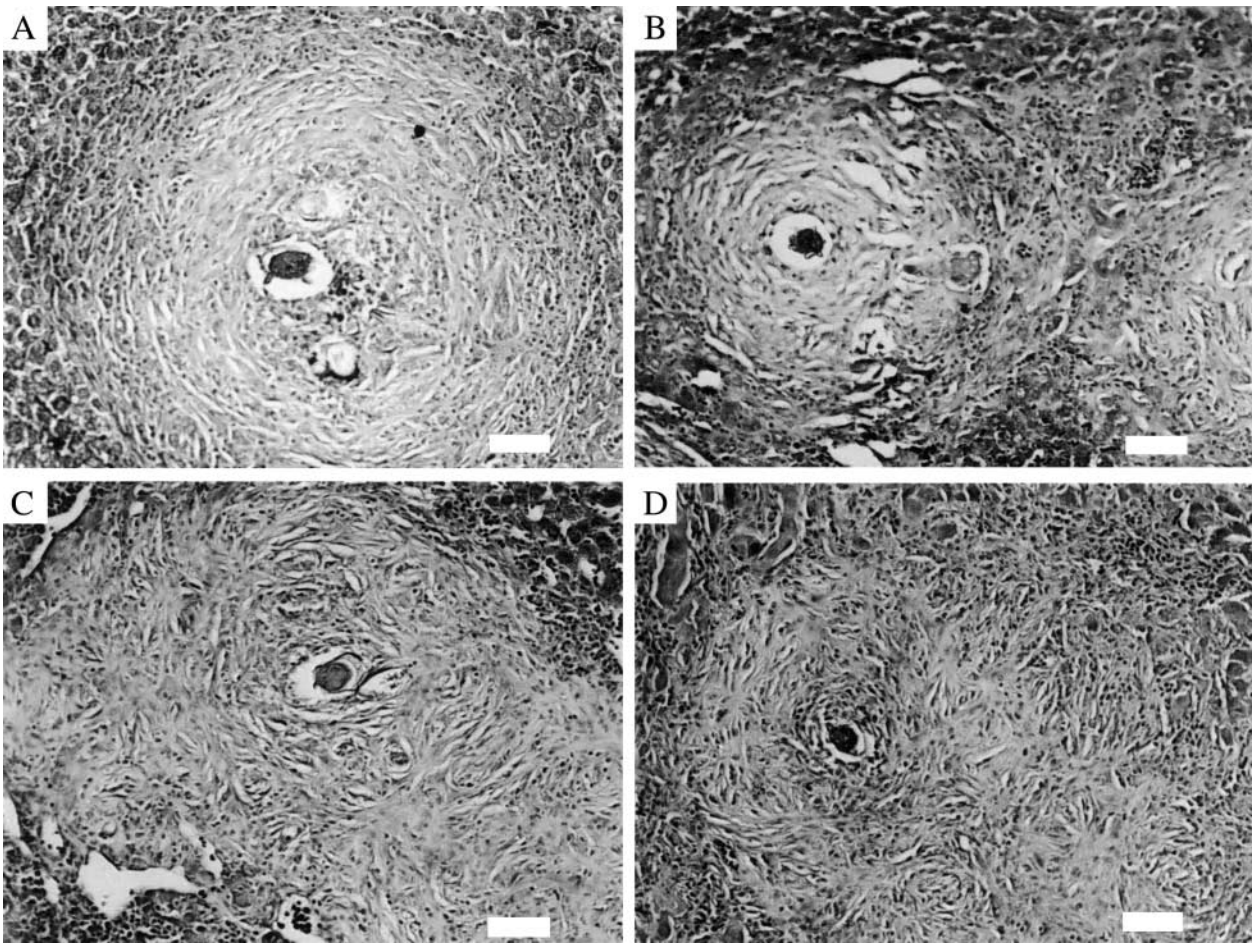

Fig. 3. Sections of schistosome egg-granulomas in the liver of mice infected with *Schistosoma mansoni* (H & E). A. Egg-granuloma in the liver of an untreated mouse infected with a PZQ-susceptible isolate. B. Egg-granuloma in the liver of a mouse infected with a PZQ-susceptible isolate on day 35 after treatment. C. Egg-granuloma in the liver of an untreated mouse infected with a PZQ-resistant isolate. D. Egg-granuloma in the liver of a mouse infected with a PZQ-resistant isolate on day 35 after treatment. Bar = 100 μm.

https://doi.org/10.1079/JOH2002135 Published online by Cambridge University Press

small number of blebs were observed on worms of the resistant isolate and most of these blebs were on the tubercles.

Twelve hours later, the number of ruptured blebs and lesions had increased on susceptible worms, and most of the tegument appeared to have been damaged. Lesions occurred over the whole surface and no normal tubercles were present (fig. 2A). The damaged tegument began to slough off in some regions. Only a few blebs and disrupted blebs were found on the surface of drug-resistant male worms, and most tubercles maintained their integrity (fig. 2B).

After 24 h, the susceptible male worm tegument appeared to have been completely damaged and parts of the tegument had been sloughed off revealing the muscle layer beneath. In contrast, resistant worms failed to reach this stage of tegumental damage, only some disrupted blebs were found and most of these were on the surface of tubercles.

Adult female worms of *S. mansoni* were more resistant to PZQ than male worms. One hour after treatment, a small amount of blebbing had begun to occur on limited areas of the anterior surface of susceptible female worms, while no blebbing was found on the surface of drug-resistant female worms. Twelve hours later, the damaged areas were enlarged and the number of blebs had increased on susceptible female worms (fig. 2C), while only a few blebs were found on a limited surface area of resistant female worms (fig. 2D). By 24 h, tegumental damage on susceptible females had become so severe that parts of the tegument were lost while no damage was found on resistant female worms.

Pathology of egg-granulomas

In untreated control groups, most of the granulomas surrounding eggs in the liver of mice infected with the three PZQ-susceptible isolates consisted of numerous infiltrating inflammatory cells, mainly eosinophils and lymphocytes and mesenchymal fibroblasts. However, in treated groups infected with PZQ-susceptible isolates on day 35 post-treatment most of the egg granulomas consisted of a small number of infiltrating inflammatory cells and mesenchymal fibroblasts. The number and size of egg-granulomas in the liver of untreated groups were larger than those in treated groups (fig. 3A,B).

Most of the egg granulomas in the liver of mice infected with the two PZQ-resistant isolates in both untreated and treated groups consisted of numerous infiltrating inflammatory cells and mesenchymal fibroblasts. There were no differences between untreated and treated groups with respect to the number and size of egg-granulomas in the liver (fig. 3C,D).

Results from analysis of the granuloma profiles showed that there were no differences between treated and untreated groups on day 35 after treatment with respect to the areas of egg-granulomas in the liver of mice infected with the two PZQ-resistant isolates However, there were significant differences between treated and untreated groups on day 35 after treatment with respect to the areas of liver egg granulomas of the three PZQ-susceptible isolates (table 1).

Discussion

We previously showed that cercariae, eggs and miracidia of putatively PZQ-resistant *S. mansoni* were less sensitive to the toxic effects of PZQ *in vitro* than drug-susceptible control isolates and that drug-resistant adult worms were less sensitive to the drug both *in vitro* and *in vivo* (Liang *et al.*, 2000, 2001). The present results are consistent with these earlier observations inasmuch as drug-susceptible *S. mansoni* worms showed progressive disruption and disintegration of the surface tegument following *in vivo* drug treatment, while putatively drug-resistant worms suffer less severe damage from equivalent drug treatment. Similar differences between drug-resistant and -susceptible worms have been described recently by William *et al.* (2001) after *in vitro* exposure of the parasites to PZQ.

The *in vitro* toxic effects of PZQ against miracidia were seen very rapidly and by 60 s after the addition of drug, resistant and susceptible miracidia could be distinguished morphologically (Liang *et al.*, 2000, 2001). The difference in size of egg-induced granulomas in the liver of mice infected with resistant and susceptible isolates could be a result of miracidia of susceptible isolates being killed more easily by the drug. The normal life span of

Table 1. Comparison of areas of egg-granulomas in the liver of mice infected with PZQ-resistant and -susceptible isolates of *Schistosoma mansoni* following treatment with 200 mg kg^{-1} PZQ on each of days 58–60 post-infection.

	Area (mean ± SD μm^2)		
Isolate of parasite	Control	Treated	*P* value
Lab-selected resistant[r]	471863.4 ± 135627.1	413806.0 ± 108971.2	0.14
Senegalese-2[r]	384325.2 ± 137503.3	400241.7 ± 225911.2	0.70
Kenya-field[s]	464097.5 ± 151087.5	292501.9 ± 99704.7	0.0001
Kenya-lab[s]	530768.3 ± 131246.0	291356.7 ± 96382.8	<0.0001
Egyptian[s]	434487.1 ± 121493.0	244300.1 ± 43829.8	<0.0001

[r] PZQ-resistant isolate.
[s] PZQ-susceptible isolate.
Measurements were made on day 35 after treatment. In each isolate, groups of ten mice were used (five for treatment, five for control), and 40 egg-granulomas were measured.

https://doi.org/10.1079/JOH2002135 Published online by Cambridge University Press

eggs in tissues is 21–30 days (Maldonado, 1959; Pellegrino *et al.*, 1962) and the egg has a pivotal role in the immunologically-mediated pathogenesis of schistosomiasis (Smithers & Doenhoff, 1982). Developed miracidia in mature eggs produce and secrete antigens that trigger the host granulomatous reaction. However, embryonic and dead eggs and egg shells do not induce granulomatous immune responses that are as pronounced as those generated by intact mature eggs (Von Lichtenberg, 1964; Pellegrino & Katz, 1969). Thus, once eggs have been killed, as for example here after drug treatment, no further antigen production takes place thus no more granulomatous reaction occurs.

A striking feature in the pathology of mice and monkeys infected with *S. mansoni* or *S. japonicum* and given curative doses of PZQ was the rapid resolution of cellular reactions and fibrosis in tissues containing eggs (Webbe *et al.*, 1981). In monkeys and rabbits infected with *S. japonicum* there was a similar resolution of pathology in the liver and bowel (Webbe *et al.*, 1981; Ying *et al.*, 1990). Effective therapy with PZQ could reduce the size of egg-granulomas surrounding eggs and reverse the magnitude of Symmers fibrosis to a certain extent (Botros *et al.*, 1984, 1986; Kumar, 1999). Mehlhorn *et al.* (1981) showed that the size of egg-granulomas in mice markedly regressed following curative treatment with PZQ: within 12 weeks their diameter had been reduced to less than half (140 μm) of the granulomas from untreated mice (340 μm). The reduction in size of egg-induced lesions appeared to be more rapid after PZQ treatment than after treatment with either stibophen or Miracil D and the relatively greater efficacy of PZQ in improving pathology may have been due to this drug having killed schistosome eggs in host tissue more effectively than the other two drugs.

Our *in vitro* tests showed that certain concentrations of PZQ could kill mature *S. mansoni* eggs collected from faeces of infected mice, but the susceptibility to PZQ of eggs from drug-susceptible isolates was significantly greater than that of eggs from resistant isolates (Liang *et al.*, 2001). Data from the pathological observations presented here show that in liver tissue *in vivo* the susceptibility of *S. mansoni* eggs to PZQ was also different between the susceptible and resistant isolates. When mice infected with PZQ-susceptible parasites were treated with $3 \times 200\,\mathrm{mg\,kg^{-1}}$ PZQ, the drug would have killed or disabled most worms and thus terminated the production of new eggs, and eggs trapped in the tissue would have been killed. Therefore, in groups of mice infected with susceptible isolates, the size of egg-granulomas in the liver of the treated mice was significantly smaller than that in untreated controls. In contrast, after treatment of mice infected with resistant isolates, the surviving worms may have continued to produce fresh eggs and a greater number of eggs already in the tissues may have survived, continued to secrete antigens and thus induced granulomatous reactions at a rate similar to that in the respective untreated control group.

This and our preceding studies (Liang *et al.*, 2000, 2001) have indicated that resistance to PZQ is manifested not only in adult worms, but also in two of the larval stages of the parasite. The fact that drug-resistance is displayed by eggs and miracidia as well as by adult worms may have consequences for the epidemiology of PZQ-resistant schistosomiasis.

Acknowledgements

This study received financial support from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. We are grateful to Mrs Kathryn A. Stafford, University of Bristol, UK for her kind assistance during the experiments and preparation of this manuscript.

References

Botros, S.S., Metwally, A.A. & Khayyal, M.T. (1984) The immunological aspects of praziquantel in unsensitized mice with experimentally induced schistosome pulmonary granuloma. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **78**, 569–572.

Botros, S.S., El-Badrawy, N., Metwally, A.A. & Khayyal, M.T. (1986) Study of some immunopharmacological properties of praziquantel in experimental schistosomiasis mansoni. *Annals of Tropical Medicine and Parasitology* **80**, 189–196.

Dunne, D.W., Bain, J., Lillywhite, J. & Doenhoff, M.J. (1984) The stage-, strain- and species-specificity of a *Schistosoma mansoni* egg antigen fraction (CEF6) with serodiagnostic potential. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **78**, 460–470.

Fallon, P.G. & Doenhoff, M.J. (1994) Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in *Schistosoma mansoni* in mice is drug specific. *American Journal of Tropical Medicine and Hygiene* **51**, 83–88.

Fallon, P.G., Sturrock, R.F., Capron, A., Niang, M. & Doenhoff, M.J. (1995) Diminished susceptibility of praziquantel in a Senegal isolates of *Schistosoma mansoni*. *American Journal of Tropical Medicine and Hygiene* **53**, 61–62.

Fallon, P.G., Mubarak, J.S., Fookes, R.E., Niang, M., Butterworth, A.E., Sturrock, R.F. & Doenhoff, M.J. (1997) *Schistosoma mansoni*: maturation rate and drug susceptibility of different geographic isolates. *Experimental Parasitology* **86**, 29–36.

Gryseels, B., Stelma, F.F., Talla, I., Van Dam, G.J., Polman, K., Sow, S., Diaw, M., Sturrock, R.F., Doehring-Schwerdtfeger, E., Kardorff, R., Decam, C., Niang, M. & Deelder, A.M. (1994) Epidemiology, immunology and chemotherapy of *Schistosoma mansoni* infections in a recently exposed community in Senegal. *Tropical and Geographical Medicine* **46**, 209–219.

Ismail, M., Metwally, A., Farghaly, A., Bruce, J., Tao, L.-F. & Bennett, J.L. (1996) Characterization of isolates of *Schistosoma mansoni* from Egyptian villagers that tolerate high doses of praziquantel. *American Journal of Tropical Medicine and Hygiene* **55**, 214–218.

Ismail, M., Botros, S., Metwally, A., William, S., Farghally, A., Tao, L.F., Day, T.A. & Bennett, J.L. (1999) Resistance to praziquantel: direct evidence from *Schistosoma mansoni* isolated from Egyptian villagers. *American Journal of Tropical Medicine and Hygiene* **60**, 932–935.

Kumar, V., (1999) (Ed.) *Trematode infections and diseases of man and animals*. Dordrecht, Kluwer Academic Publishers.

Liang, Y.-S., Coles, G.C. & Doenhoff, M.J. (2000) Detection of praziquantel resistance in schistosomes. *Tropical Medicine and International Health* **5**, 72.

Liang, Y.-S., Coles, G.C., Doenhoff, M.J. & Southgate, V.R. (2001) *In vitro* responses of praziquantel-resistant and -susceptible *Schistosoma mansoni* to praziquantel. *International Journal for Parasitology* **31**, 1227–1235.

Maldonado, J.F. (1959) The longevity of the unhatched miracidium of *Schistosoma mansoni* in the tissues of mice. *American Journal of Tropical Medicine and Hygiene* **8**, 16–19.

Mehlhorn, H., Becker, B., Andrews, P., Thomas, H. & Frenkel, J.K. (1981) *In vivo* and *in vitro* experiments on the effects of praziquantel on *Schistosoma mansoni*, a light and electron microscopic study. *Arzneimittel-Forschung/Drug Research* **31**, 544–547.

Pellegrino, J. & Katz, N. (1969) Laboratory evaluation of antischistosomal agents. *Annals of the New York Academy of Sciences* **160**, 429–460.

Pellegrino, J., Oliveira, C.A., Faria, J. & Cunha, A.S. (1962) New approach to the screening of drugs in experimental *Schistosoma mansoni* in mice. *American Journal of Tropical Medicine and Hygiene* **11**, 201–215.

Radke, M.G., Berrios-Durran, L.A. & Moran, K. (1961) A perfusion procedure for recovery of schistosome worms. *Journal of Parasitology* **47**, 366–368.

Smithers, S.R. & Doenhoff, M.J. (1982) Schistosomiasis. pp. 527–607 *in* Warren, S. & Warren, K.S. (*Eds*) *Immunology of parasitic infections*. Oxford, Blackwell Scientific.

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

Sobhon, P. & Upatham, E.S. (1990) Effect of praziquantel on adult oriental schistosomes. pp. 247–270 *in* Sobhon, P. & Upatham, E.S. (*Eds*) *Snails hosts, life-cycle and tegumental structure of oriental schistosomes*. Bangkok, Lincoln Promotion.

Standen, O.D. (1949) Experimental schistosomiasis. II. Maintenance of *Schistosoma mansoni* in the laboratory, with some notes on experimental infection with *S. haematobium*. *Annals of Tropical Medicine and Parasitology* **43**, 268–283.

Stelma, F.F., Talla, I., Sow, S., Kongs, A., Niang, M., Polman, K., Deelder, A.M. & Gryseels, B. (1995) Efficacy and side effects of praziquantel in an epidemic focus of *Schistosoma mansoni*. *American Journal of Tropical Medicine and Hygiene* **53**, 167–170.

Von Lichtenberg, F. (1964) Studies on granuloma formation III: antigen sequestration and destruction in the schistosome pseudotubercle. *American Journal of Pathology* **45**, 75–93.

Webbe, G., James, C., Nelson, G.S. & Sturrock, R.E. (1981) The effect of praziquantel on *Schistosoma haematobium*, *S. japonicum* and *S. mansoni* in primates. *Arzneimittel-Forschung/Drug Research* **31**, 542–544.

William, S., Botros, S., Ismail, M., Farghally, A., Day, T.A. & Bennett, J.L. (2001) Praziquantel-induced tegumental damage *in vitro* is diminished in schistosomes derived from praziquantel-resistant infections. *Parasitology* **122**, 63–66.

World Health Organization (1993) The control of schistosomiasis. Second report of the WHO expert committee. *World Health Organization Technical Report Series* **830**.

Ying, Y.Y., Lei, X.X. & Yang, Y.Q. (1990) Pathology. pp. 331–369 *in* Mao, S.B. (*Ed.*) *Schistosome biology and control of schistosomiasis*. Beijing, People Health Publishing House.

(Accepted 28 June 2002)