The effect of praziquantel treatment on glutathione concentration in *Schistosoma mansoni*

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SUMMARY

A fluorescent dye monochlorobimane (MCB) that binds glutathione (GSH) was used as a tool for measuring the concentration of GSH in skin and mechanically-transformed schistosomula. The specificity of MCB binding to GSH was confirmed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The MCB binding to GSH is an energy-dependent process since no labelling could be seen at low temperature. When 24-h-old schistosomula were depleted of GSH by buthionine sulfoximine (a specific inhibitor of GSH synthesis) for 18 h, a significant decrease ($P < 0{\cdot}001$) in fluorescence was observed. PZQ treatment of the schistosomula after first labelling the parasites with MCB did not greatly affect MCB binding to GSH. However, when the 24-h-old schistosomula were first PZQ treated and afterwards labelled with MCB, the pattern of labelling was identical to that of those of the non-labelled parasites. When 24-h-old schistosomula were first PZQ treated, washed and labelled in the presence of 1 mM GSH, the level of fluorescence was recovered. These results suggest that PZQ depletes GSH from schistosomula, and may render them susceptible to the host's immune system.

Key words: *Schistosoma mansoni*, praziquantel, glutathione, monochlorobimane, buthionine sulfoximine.

INTRODUCTION

Glutathione (GSH), a tripeptide synthesized intracellularly is known to take part in many important biological processes such as DNA and protein synthesis, enzyme activity, transport and protection of cells (Meister & Anderson, 1983). GSH is a crucial reducing agent and antioxidant involved in the maintenance of the optimal cellular oxidation-reduction balance, an essential condition for cellular survival. It can react with a large number of foreign compounds that have an electrophilic centre to form GSH conjugates. The GSH interaction with foreign compounds may be either spontaneous or catalysed by glutathione S-transferase (GSH S-transferase).

Studies concerning both GSH and GSH-S transferase have been carried out in different model systems since they have a pivotal role in the course of cancer and parasitic diseases. The conjugation reactions of GSH to xenobiotics are regarded as detoxification processes which are key defence mechanisms used by cells to prevent accumulation and toxicity of exogenous and endogenous compounds (Ishikawa, 1992). Arrick, Griffith & Cerami (1981) have demonstrated that inhibition of GSH biosynthesis can render blood-stream forms of *Trypanosoma brucei* susceptible to hydrogen peroxide (H_2O_2) damage.

Mkoji, Smith & Pritchard (1989) have demonstrated a decrease in reduced glutathione (GSH) levels in *Schistosoma mansoni* adult worms exposed *in vitro* to Oltipraz (OPZ), an anti-schistosomal drug, and this result was accompanied by a significant increase in oxidized glutathione (GSSG) levels. Praziquantel (PZQ) is the drug of choice for human schistosomiasis treatment but its mechanism of action has not been precisely clarified. It is well known from the literature that PZQ induces Ca^{2+} influx in schistosomes causing a fast and subsequent muscular contraction (Redman *et al*. 1996). PZQ also reveals hidden antigens, on the schistosome surface, which are targets to the host immune response (Harnett & Kusel, 1986; Brindley *et al*. 1989). However, the possible effect of PZQ in *S. mansoni* on GSH levels has not been determined.

To assess where GSH is located in *S. mansoni* and to characterize whether anti-schistosomal activity of PZQ is related to reduction of parasite GSH levels, we have used monochlorobimane (MCB), a probe of

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choice to detect GSH in studies using mammalian cells as a model. MCB is a non-fluorescent hydrophobic compound that permeates into cells, reacts with GSH yielding a fluorescent GSH-bimane adduct. Intracellular conjugation is exclusively mediated by glutathione S-transferase although there is a very slow spontaneous formation of the adduct (Shrieve, Bump & Rice, 1988). The rate of conjugation between GSH and MCB has been demonstrated to be correlated with cellular compositions of GST isoenzymes (Ublacker *et al*. 1991).

MATERIALS AND METHODS

Parasites and hosts

A Puerto Rican strain of *S. mansoni* was routinely maintained in TO mice and *Biomphalaria glabrata* snails. Schistosomula obtained by mechanical transformation of cercariae by the syringe method of Colley & Wikel (1974) were cultured for 24 h in Glasgow modification of Eagle's medium (GMEM, Gibco Limited, Paisley, Scotland) supplemented with heat-inactivated foetal calf serum (FCS, Sigma Chemical Co.) plus 100 IU/ml penicillin–100 μg streptomycin (Gibco, BRL) and 2 mM L-glutamine (Gibco, BRL) at 37 °C in a 5% CO_2 incubator. When experiments with buthionine sulfoxine (BSO) were carried out, the drug was added to the culture medium for the final 18 h of the 24 h culture period. Skin-transformed schistosomula were obtained by the method of Clegg & Smithers (1972) and incubated as above. Parasites were washed 3 times in warm GMEM before labelling to remove FCS.

Fluorescent probes and drugs

Monochlorobimane (syn-($ClCH_2$, CH_3)-1,5-diazabicyclo-[3.3.0]octa-3,6-dione-2,8-dione; MCB) was purchased from Molecular Probe Inc., Eugene, OR, USA. A stock solution of 1 mM MCB was prepared in ethanol, protected from light and stored at −25 °C. Parasites were labelled with MCB at 10 μM final concentration in all experiments. Praziquantel (PZQ) was a gift from Achim Harder (Bayer AG, Wuppertal, Germany). A stock solution of 50 mg/ml in ethanol was used. Buthionine sulfoximine (BSO) was purchased from Sigma Chemical Co., Poole, Dorset. A 100 mM stock solution of BSO was prepared in GMEM medium and kept below −25 C.

Quantitative fluorescence

Fluorescence intensity, of skin-transformed and mechanically-transformed schistosomula labelled with MCB, was quantified on a MPV attachment to a Leitz Ortholux microscope. The MCB probe has maximum absorbance at 380 nm and maximum emission at 461 nm. All photographs were taken using an Ortholux II microscope with a camera attached and an ASA 1600 Ektachrome colour film was used. The parasites were immobilized for photography with 1 mg/ml carbachol (Sigma Chemical Co., Poole, Dorset). Carbachol did not influence the distribution of fluorescence.

Thin layer chromatography

Thin layer chromatography was carried out to separate MCB from its adducts, and to identify adducts formed within the schistosomula. Silica gel plastic backed thin layer chromatography plates (Merck, Darmstadt, Germany) were used with the solvent propanol/H_2O/acetic acid (8:6:1) by the method of Oude Elferink *et al*. (1993). MCB and its adducts were detected using a hand-held U.V. lamp (USL Lamp, Minnesota, USA). Standard glutathione-MCB adducts were made by adding 1 U glutathione S-transferase (Sigma Chemical Co., Poole, Dorset) to 1·0 ml of GMEM containing 500 μM glutathione and 50 μM MCB. The reaction was allowed to continue for 1 h after which no further increase in fluorescence occurred (Oude Elferink *et al*. 1993).

High performance liquid chromatography

Analysis was carried out using a Thermoseparations P100 HPLV pump fitted with a Rheodyne injector with a 20 μl loop. A Hypersil 5 Phenyl column (250 × 4·6 mm, Phenomenex UK) was used, the mobile phase was methanol/0·02 M potassium phosphate buffer, pH 6·5 (25:75), with a flow rate of 1 ml/min. Detection was carried out with a Shimadzu RF-530 fluorescence detector with excitation set at 390 nm and emission monitored at 490 nm, the signal was outputed to a Hewlett-Packard 3395 integrator.

Statistical analysis

The results were analysed by Student's *t*-test.

RESULTS

Glutathione distribution in schistosomula

Twenty-four h-old mechanically transformed schistosomula were incubated with 10 μl of a stock solution of MCB (1 mM) in 1·0 ml of medium for 1 h at 10 μM final concentration. The pattern of labelling of the schistosomula, under these conditions, showed fluorescence localized in the glands,

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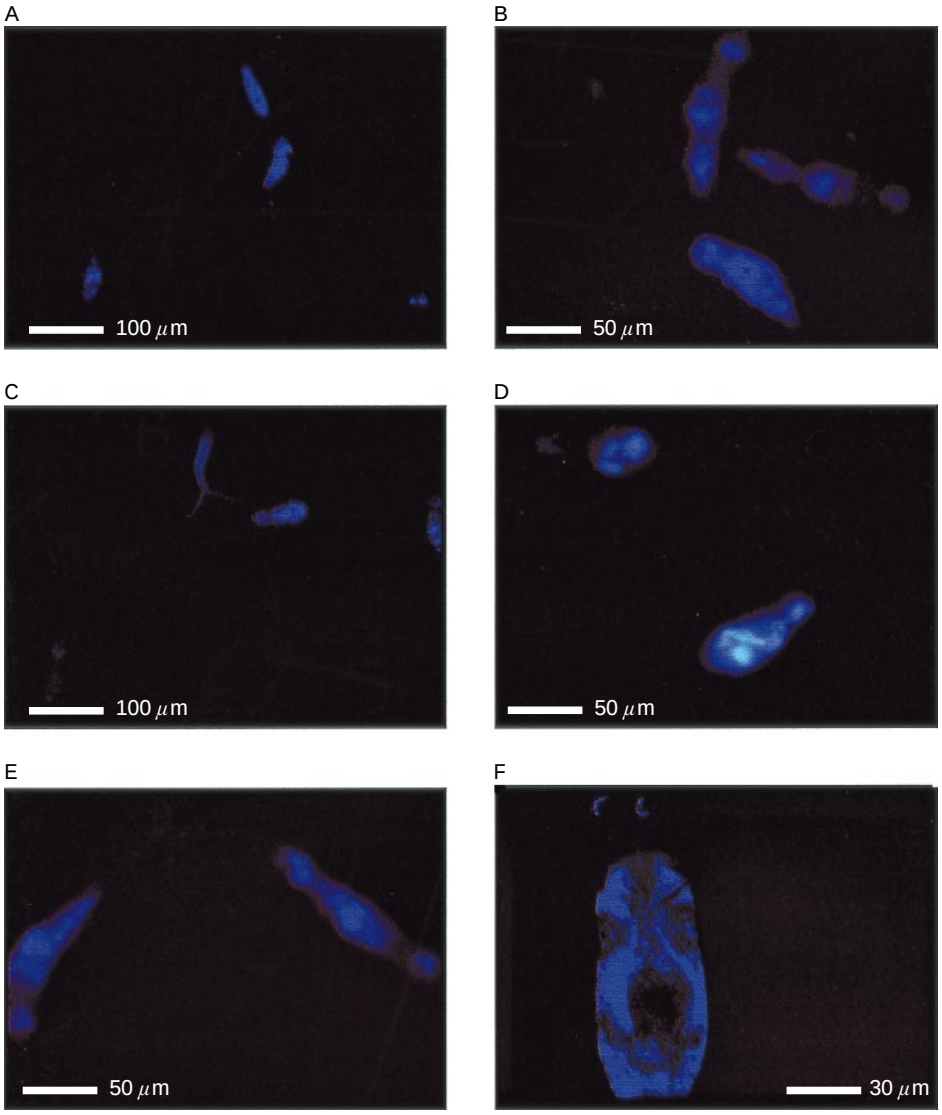

Fig. 1. (A) Mechanically-transformed schistosomula incubated in buthionine sulfoximine for 18 h, and then stained with monochlorobimane (MCB). (B, C) Mechanically-transformed schistosomula treated with praziquantel (25 μg/ml) and then stained with MCB. (D) Mechanically-transformed schistosomula stained with MCB. (E) Skin-transformed schistosomula stained with MCB. (F) Mechanically-transformed schistosomula stained with MCB to show granular nature of staining in the glands.

gland ducts and in the cytoplasm of the parenchyma cells (Fig. 1 A). Some variability in dye concentration in the glands of the schistosomula was observed as well. Skin-transformed schistosomula showed little labelling in the glands but evident labelling in the parenchyma cells (Fig. 1 E).

Effect of temperature on MCB binding

To evaluate whether dye binding to GSH is a temperature-dependent process, 24-h-old schistosomula were labelled with MCB for 1 h at 4 °C. After this time, the parasites were washed in medium at 4 °C. No MCB binding was observed since the fluorescence emission detected was the same as that of the background level. This background level was measured on unlabelled parasites and was due to autofluorescence. Even when the parasites, labelled at 4 °C, were warmed to 37 °C for 1 h or overnight incubation, no recovery of fluorescence was detectable (Fig. 2). This finding suggests that MCB binding to GSH is an energy-dependent process and that the very little non-specific binding that occurred in glands and parenchyma was temperature independent.

Glutathione depletion prevents binding of MCB

To assess whether MCB is binding specifically to GSH in the *S. mansoni* model, 24-h-old schistosomula were previously incubated with 2 different concentrations (50 μM and 500 μM/ml) of a specific GSH synthesis inhibitor buthionine sulfoximine (BSO) for 18 h at 37 °C. A significant decrease in

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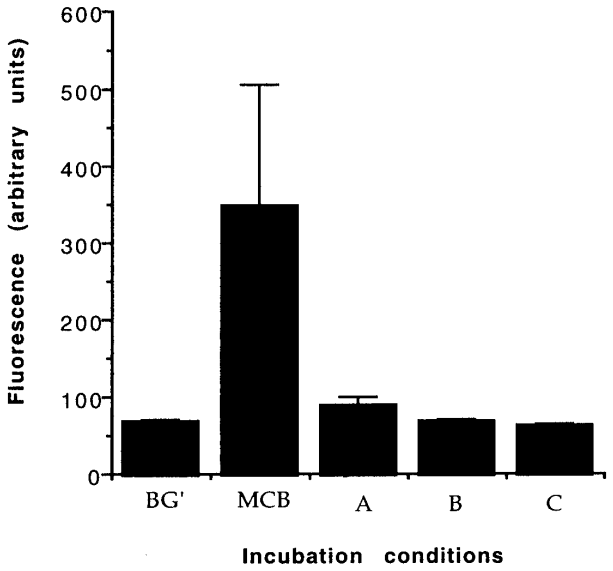

Fig. 2. 24-h-old schistosomula were labelled with MCB for 1 h at 37 °C in GMEM medium under different conditions. (A) 24-h-old schistosomula labelled with MCB for 1 h at 37 °C in GMEM medium. (B) 24-h-old schistosomula labelled with MCB for 1 h at 4 °C in GMEM medium, washed and warmed to 37 °C for 1 h. (C) 24-h-old schistosomula labelled with MCB for 1 h at 4 °C in GMEM medium, washed and warmed to 37 °C overnight. BG′ represents auto-fluorescence background of unlabelled parasites.

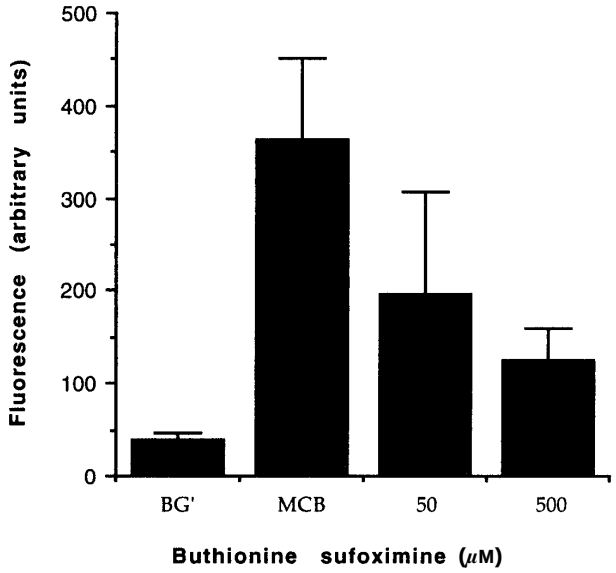

Fig. 3. Fresh mechanically-transformed schistosomula were incubated for 18 h at 37 °C in GMEM medium, in the presence of different concentrations of BSO. After 18 h incubation, parasites were washed in GMEM medium and labelled with MCB for 1 h at 37 °C. BG′ represents autofluorescence background of unlabelled parasites.

MCB fluorescence ($P < 0{\cdot}001$) was observed (Fig. 3). Although schistosomula incubated with the lower BSO concentration (50 μM) demonstrated a significant decrease in fluorescence level, a mild fluorescence could be seen in the glands. However,

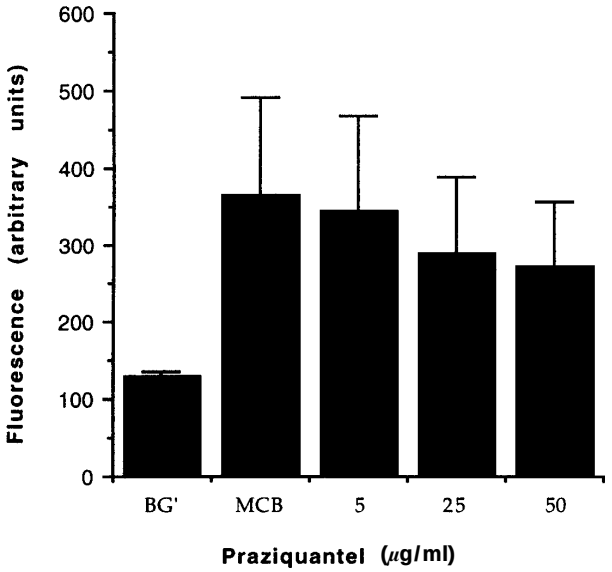

Fig. 4. 24-h-old schistosomula were labelled with MCB for 1 h at 37 °C in GMEM medium, washed in a probe-free GMEM medium and afterwards treated with different concentrations of PZQ for 1 h at 37 °C in GMEM medium.

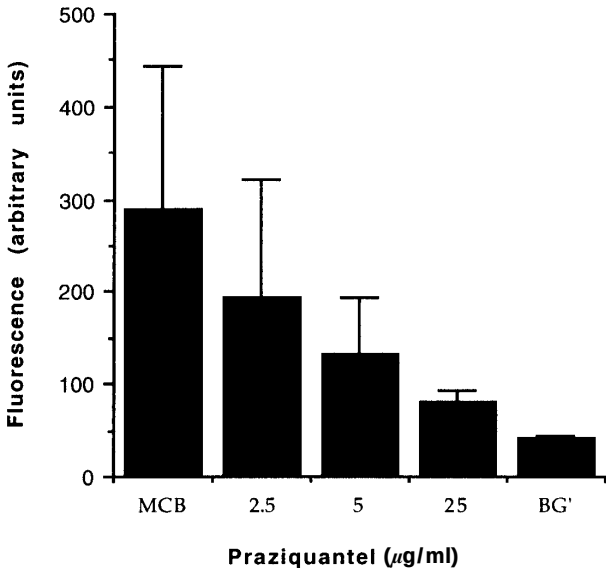

Fig. 5. 24-h-old schistosomula were first treated with different concentrations of PZQ for 1 h, washed and labelled with MCB for 1 h at 37 °C in GMEM medium. After labelling, parasites were washed in probe-free GMEM medium. BG′ represents autofluorescence background of unlabelled parasites.

the schistosomula incubated with 500 μM BSO showed almost the same pattern of background fluorescence as unlabelled parasites, with only few parasites showing a faint labelling in the glands.

PZQ treatment inhibits MCB binding

Twenty-four h-old schistosomula were labelled with MCB for 1 h at 37 °C, washed and subsequently treated with different PZQ concentrations (5, 25 and 50 μg/ml) for 1 h at 37 °C. The MCB binding to

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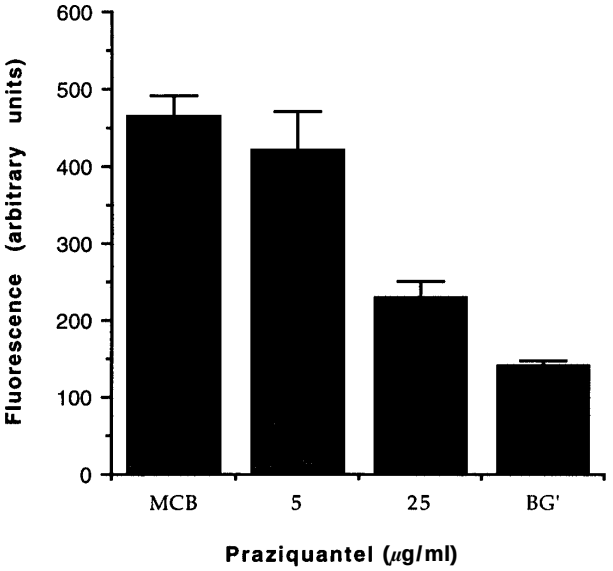

Fig. 6. Skin-transformed schistosomula were first treated with different concentrations of PZQ for 1 h, washed and labelled with MCB for 1 h at 37 °C in GMEM medium. After labelling, parasites were washed in probe-free GMEM medium. BG′ represents autofluorescence background of unlabelled parasites.

GSH measured by fluorescence emission of the dye from the labelled parasites was not reduced by this treatment (Fig. 4). However, when schistosomula were first treated with PZQ (2·5, 5 and 25 μg/ml), washed and subsequently labelled with MCB a significant decrease in fluorescence emission was observed. The PZQ pre-treatment of the parasites with 5 and 25 μg caused a strong decrease ($P <$ 0·001) in MCB binding (Fig. 5). When skin-transformed schistosomula were treated with 2 different PZQ concentrations (5 and 25 μg/ml), washed and then labelled with MCB, a significant decrease in fluorescence was also observed. These results suggest that PZQ may be interfering with GSH levels in the parasite. (Fig. 6).

In order to see whether GSH addition could restore MCB labelling in schistosomula previously treated with 25 μg PZQ, 24-h-old schistosomula were incubated in presence of 1 μM GSH for 1 h, washed and labelled with 10 μM MCB. As shown by Fig. 7, schistosomula labelling was recovered by GSH addition.

Effect of GSH depletion on PZQ activity

To see whether GSH depletion could enhance damage caused by PZQ, 24-h-old schistosomula were previously cultured in the presence of 500 μM BSO for 18 h and treated with different PZQ concentrations (2·5, 5, 10 and 25 μg/ml). All GSH-depleted groups showed enhanced PZQ-caused damage, with more blebs and inner structures being disorganized when compared with PZQ-treated groups not GSH-depleted (Fig. 8).

Thin-layer chromatography analysis of MCB adducts

Extracts of schistosomula labelled with MCB in propanol/H_2O/acid acetic (8:6:1) were applied to the thin layer chromatography plate, beside MCB and MCB-glutathione standards. After chromatography in the above solvent, it could be seen that schistosomula possessed 1 major spot co-migrating with MCB-glutathione. A small amount of fluorescence was observed at the origin and this perhaps represents protein-bound MCB. The band co-migrating with MCB-glutatione was scraped off the plate and eluted with 20 mM phosphate buffer (pH 6·5) in 25 % methanol.

High performance liquid chromatography

It can be seen that the major schistosome product gave a trace identical to that of the standard glutathione-MCB adduct. Fig. 9 shows the HPLC traces corresponding to the standard MCB-GSH adduct and the schistosome extract, after elution from the thin layer chromatography plate.

DISCUSSION

In this article we have shown that MCB is a useful probe to detect GSH in *Schistosoma mansoni*. MCB fluorescence was found to localize in the glands, gland ducts and parenchyma cells of 24-h-old schistosomula whereas skin-transformed schistosomula demonstrated fluorescence only in the parenchyma cells, since all glands have evacuated.

The binding of MCB to GSH in 24-h-old schistosomula is an energy-dependent process since when the parasites were labelled at 4 °C no fluorescence was detected. When the 4 °C-labelled schistosomula were warmed to 37 °C for 1 h or for overnight incubation, no difference from the background parasites (not labelled and observed under the 475–485 filter to detect autofluorescence) was observed.

Buthionine sulfoximine (BSO) is a potent and specific inhibitor of GSH synthesis (Griffith & Meister, 1979; Griffith, 1982). The compound is highly active as an inhibitor of γ-glutamylcysteine synthetase *in vitro* and *in vivo* (Griffith & Meister, 1979). In order to see whether MCB binds specifically to schistosomula GSH, we labelled parasites previously incubated with 2 different BSO concentrations. As shown, a significant reduction in fluorescence, 67 % to control, was observed in GSH-depleted parasites with 500 μM BSO. It should be noticed that the time (18 h) of incubation or the BSO concentration here used may be not enough to allow total GSH depletion in 24-h-old schistosomula. However, the results observed here suggest that MCB is binding specifically to GSH in *S. mansoni*

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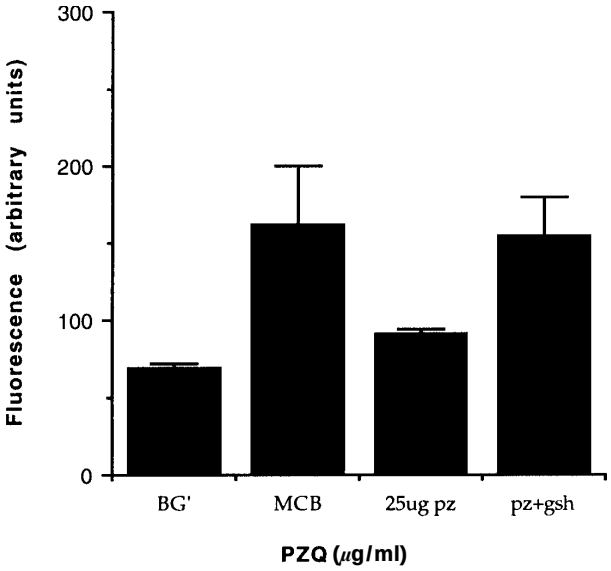

Fig. 7. 24-h-old schistosomula were labelled with MCB for 1 h at 37 °C in GMEM medium (second bar) or were first treated with 25 μg PZQ for 1 h, washed and labelled with MCB for 1 h at 37 °C in GMEM medium (third bar) or were first treated with 25 μg PZQ for 1 h, washed and labelled with MCB, for 1 h at 37 °C in GMEM medium, in the presence of 1 μM GSH. BG′ represents autofluorescence background of unlabelled parasites.

When extracts of MCB-labelled schistosomula were separated by thin layer chromatography, a band running at the same position as MCB-glutathione was the major component. The identity of this band as MCB-glutathione was confirmed by HPLC.

Although the mode of action of PZQ has been extensively studied there is no evidence that it alters the concentration of GSH in *S. mansoni*. Here, we have shown that when 24-h-old schistosomula were labelled with MCB and subsequently treated with different PZQ concentrations only a slight decrease in fluorescence was observed. The result above suggests that once MCB is bound to schistosomular GSH and PZQ reaction does not affect the quantity of adduct. However, when 24-h-old schistosomula were first treated with PZQ and afterwards labelled with MCB, a marked reduction in fluorescence ($P < 0{\cdot}001$) was demonstrated. These results suggest that PZQ could either be provoking GSH release from the parasites, thus impairing MCB binding or PZQ could be reacting with glutathione S-transferase, the enzyme required to catalyse the MCB binding reaction to GSH. McTigue, Willams & Tainer (1995) have shown, using X-ray crystallography, that a complex can be formed between glutathione S-transferase from *S. japonicum* and PZQ. However,

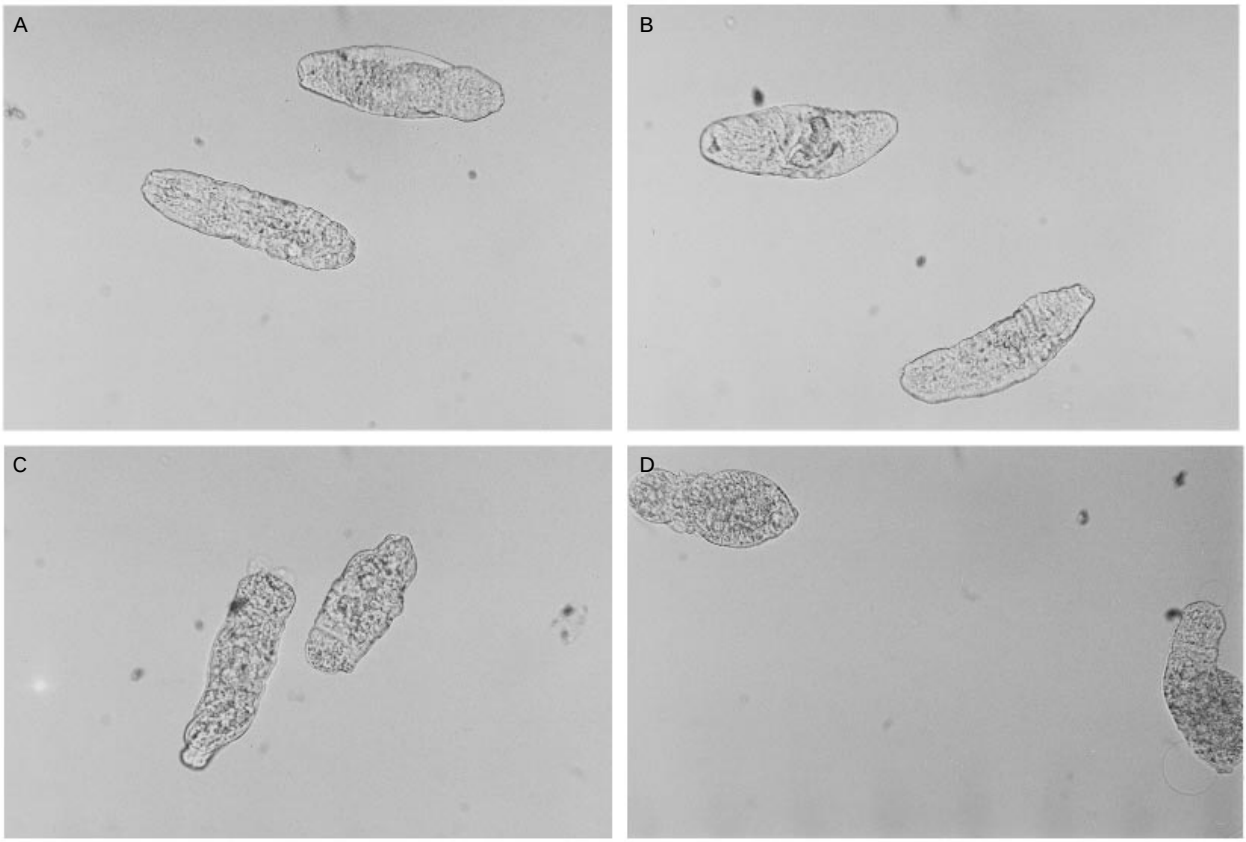

Fig. 8. (A) Schistosomula not depleted of glutathione. (B) Schistosomula depleted of glutathione with BSO. (C) Schistosomula not depleted of glutathione, treated with PZQ (10 μg/ml). (D) Schistosomula depleted of glutathione with BSO, treated with PZQ (10 μg/ml).

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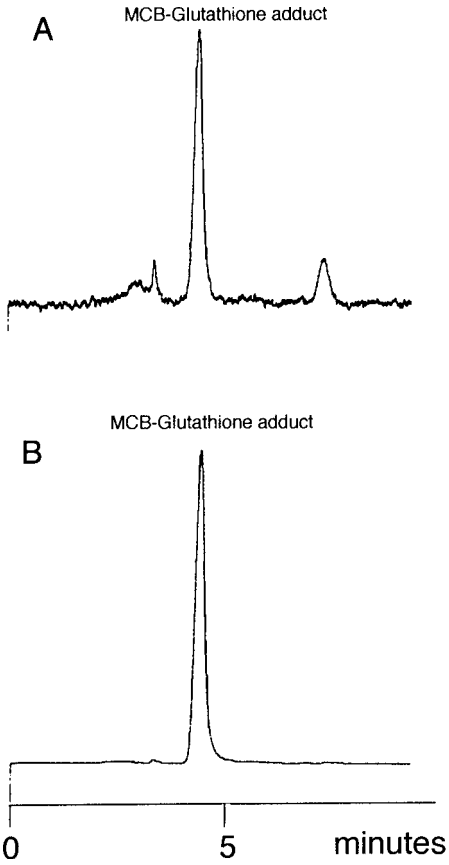

Fig. 9. (A) HPLC trace corresponding to glutathione-MCB standard extracted from schistosomes. The spot corresponding in Rf to the standard was scraped from a TLC plate and eluted with methanol/0·02 M potassium phosphate buffer, pH 6·5, prior to analysis by HPLC with fluorescence detection. (B) Trace obtained for the enzymatically prepared glutathione-MCB standard treated in the same way.

Walker *et al*. (1993), working with a model of recombinant Sm28GST or Sj26GST, could not detect any inhibition of either enzyme using up to 500 μM PZQ concentration.

It should be noted that the concentrations of PZQ used in these studies may be larger than the blood levels of infected mice of humans following treatment.

When schistosomula GSH-depleted by BSO were treated with PZQ, each GSH-depleted group showed enhanced damage depending on the PZQ dose when compared with the respective control group not GSH depleted as assessed by light microscopy. This is indicative that GSH may be important in parasite protection against damage caused by drug treatment. Thus PZQ seems to be interfering in this possible protective activity of GSH in schistosomula. Treatment of schistosome-infected hosts with PZQ may also allow the host's immune system to be more effective against the parasite because of depletion of glutathione levels. Such possibilities are being investigated.

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