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Identification and localization of glutathione S-transferase as a potential target enzyme in *Brugia* species

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Abstract *Brugia* filarial nematodes are pathogenic lymphatic-dwelling parasites that, like other helminths, may modify the host's defense mechanisms by a major detoxification process involving glutathione-binding proteins such as glutathione S-transferases (GSTs). In the present study, soluble extracts of third-stage larvae, adult male and female worms, microfilariae of either *B. pahangi* or *B. malayi* or the adult worm excretory–secretory products of *B. malayi* were used to determine GST activity. These extracts and affinity-purified fractions of *B. pahangi* adult worms had a specific enzymatic activity when 1-chloro-2,4-dinitrobenzene was used as a substrate. The observance of this enzyme in all life cycle stages of *Brugia* spp. demonstrates its ubiquitous nature. Lavage of intraperitoneally infected jirds, but not that of uninfected jirds, also showed increased enzymatic activity, suggesting that GST is secreted in vivo. Soluble proteins of both *Brugia* spp. were strongly recognized by antibodies in sera from rabbits immunized with affinity-purified native GST of *Onchocerca volvulus*. Immunohistochemical studies localized these proteins in adult worms, demonstrating cross-reactivity between the GST of these two filarial nematodes. The effect of this enzyme on the motility and viability of adult worms, microfilariae, and larvae was tested in vitro using a battery of known GST inhibitors. Of all those tested, ethacrynic acid, *N*-ethylmalemide, 4-nitropyridine-oxide, or 1-chloro-2,4-dinitrobenzene at micromolar concentrations reduced the viability and motility of microfilariae, third-stage larvae, and adult worms. These results suggest that *Brugia* GSTs are major metabolic enzymes and may play an important role in the parasite's survival.

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Introduction

The filarial worms *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*, the etiological agents of lymphatic filariasis, infect more than 120 million people (Michael et al. 1996; Ottesen et al. 1997); and several million others are at risk for acquiring such infection if control measures fail to meet the standards set by the World Health Organization. Therefore, it is imperative to identify the key enzymes and biochemical pathways that are pivotal to the parasites' survival in the host's hostile environment, including their oxidative stresses and immune responses. These enzymes should provide excellent biochemical targets for developing effective chemotherapies and vaccines (Grieve et al. 1995; Lazdins and Kron 1999). One such enzyme is glutathione S-transferase (GST; EC 2.5.1.18; Brophy and Pritchard 1994), which is involved in xenobiotic metabolism, intracellular binding, and biosynthesis of endogenous substrates such as prostaglandins and leukotrienes (Boyer 1989; Armstrong 1991; van Bladeen and van Ommen 1991). GSTs may potentially favor parasite survival by neutralizing the toxins acting against them and may repair host-induced damage (Mitchell 1989). GSTs are divided into four major classes (alpha, mu, pi, and theta) and a microsomal class; and they occur in varying quantities in different mammalian tissues (Mannervik et al. 1985; Morgenstern et al. 1985; Warholm et al. 1986). Specific inhibitors for

some of these classes have been identified (Tahir and Mannervik 1985; Ploemen et al. 1990). GST isozymes, which have very little homology with some of these subclasses, have also been found in helminths, including filarial worms (Balloul et al. 1987; Weston et al. 1989; Trottein et al. 1990; Panaccio et al. 1992; Liebau et al. 1994a, c; Salinas et al. 1994). Because GSTs are major detoxifying enzymes in helminths, they may be able to scavenge the products of lipid peroxidation and to metabolize toxic products, including anthelmintics. These biological functions make GSTs molecular targets for new antifilarial drugs (Brophy and Pritchard 1994). GST isozymes have shown potential as protective antigens against fascioliasis and schistosomiasis (Balloul et al. 1987; Mitchell 1989; Sexton et al. 1990; Boulanger et al. 1991; Capron et al. 1992; Xu et al. 1993).

In this report we describe the presence of GST activity in worm extracts. We observed immunoreactivity of *Brugia* antigens to antisera of *Onchocerca volvulus* GST. Immunolocalization of these proteins in worm tissues suggests that they may have cross-reactive epitopes. The ubiquitous nature of GSTs in the life cycle stages of *Brugia* spp. and the effect of various inhibitors on worm viability and motility in vitro indicate that this enzyme could be a molecular target for the next generation of drugs against filarial nematodes.

Materials and methods

Animals and infections

Brugia infections were routinely maintained in our laboratory in jirds (*Meriones unguiculatus*) obtained from Tumblebrook Farms (West Brookfield, Mass.) and *Aedes aegypti* mosquitoes. Infected animals were fed on rodent chow and water ad libitum and treated according to the Principles of Laboratory Care (NIH Publication No. 86-23) with approval by the Louisiana Animal Medical Ethics Committee, School of Veterinary Medicine, Louisiana State University.

Parasites and extracts

Adult male and female worms were obtained from the peritoneal cavities of infected female jirds at >60 days and >120 days post-infection (DPI) for *B. pahangi* and *B. malayi,* respectively. Fourth-stage larvae (L4) were recovered from jirds in a similar manner at 14 DPI. Infective third-stage larvae (L3) were obtained from *A. aegypti* mosquitoes fed 11 days earlier on microfilaremic jirds. Microfilariae (Mf) recovered by lavaging the peritoneal cavity of intraperitoneally infected jirds were isolated by density gradient centrifugation using Histopaque (Sigma, St. Louis, Mo.). Parasites at all life cycle stages were washed three times with RPMI-1640 to eliminate host-cell contamination and used immediately for protein extraction. Soluble extracts of mixed male and female worms, single sexed worms, Mf and L3 of *B. malayi* or *B. pahangi* were prepared individually in phosphate-buffered saline (PBS), pH 7.2, as described by Rao et al. (1996) and stored at −70 °C until use.

Excretory–secretory (ES) products of mixed worms of *B. malayi* were generated by incubating 100 female and 100 male worms in 50-ml culture flasks containing medium (RPMI-1640 supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 100 μg ml^{-1} penicillin, 100 U ml^{-1} streptomycin, and 5% fetal calf serum. Worms were placed in fresh medium every 48 h and old medium was collected. Worms were cultured for 7 days with 100% viability. The cultured media were pooled and concentrated with ultrafiltration units (3000 MW cutoff, Amicon, Bedford, Mass.). The concentrate was dialyzed against ice-cold PBS and stored at −70 °C until use.

Protein concentrations of soluble extracts and ES products were estimated by Bio-Rad reagents (Bio-Rad, Hercules, Calif.) or BCA-protein detection test (Pierce, Rockford, Ill.). The soluble extracts were passed through a 0.22-μm millipore filter, aliquoted, and stored at −70 °C.

Isolation of *Brugia* glutathione-binding proteins and enzyme assay for GST

Soluble adult worm extract of *B. pahangi* in cold PBS was centrifuged at 100,000 *g* for 30 min. The glutathione-binding protein(s) was further purified from the soluble fraction by affinity chromatography on a GSH-coupled-Sepharose 4B column (Sigma; Simons and Vander Jagt 1971; Habig and Jakoby 1981). Enzyme assay for GSTs was performed spectrophotometrically using the extracts of Mf, male, female, L3, L4, or a mixture of male and female worms, ES, or affinity purified glutathione-binding proteins and 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Habig et al. 1974; Jaffe and Lambert 1986). A typical reaction mixture for assay of GST contained the parasite protein, reduced GSH (2.5 mM), and CDNB (1.0 mM). Selected parasite protein in phosphate buffer (pH 6.5) was incubated for 3 min at room temperature. GSH and CDNB were added and the reaction was monitered as an increase in absorbance at 340 nm (A_{340}) for 3 min. For comparison, a cytosol fraction of jird liver known to have high GST activity was isolated from uninfected age-matched control jirds and tested in this system as positive controls. Peritoneal lavage from uninfected and infected jirds with L3 (>100 DPI) was collected in ice-cold PBS and centrifuged at 10,000 *g* for 30 min. The supernatants from these samples were also tested for GST activity. GST profiles in the first five fractions were determined by resolving a 15-μl sample on a 15% SDS gel under reducing conditions and further staining by silver (Salinas 1994).

Isolation of *Onchocerca volvulus* glutathione-binding proteins and immunization of rabbits

Briefly, female *Onchocerca volvulus* adult worms collected from Sierra Leonean patients were made into powder form and resuspended in PBS. The soluble supernatant was passed through a glutathione agarose column; and the GST proteins were eluted with PBS and 1% Triton x-100 containing 5 mM glutathione. Rabbits were immunized with this affinity-purified GST protein (a pooled fraction of either a 24-kDa doublet protein or a mixture of a 24-kDa doublet and 32-kDa and 36-kDa proteins) as described earlier (Salinas et al. 1994). Serum was collected 2 weeks after the booster injection and stored at −70 °C until use. Serum from rabbit immunized with 24-kDa doublet was designated ab-A and that from rabbit immunized with the mixture of 24-kDa doublet, 32-kDa and 36-kDa was designated ab-B.

Enzyme-linked immunosorbent assay to determine GST in *Brugia* extracts

For enzyme-linked immunosorbent assay (ELISA), flat-bottomed polyvinyl microtiter plates were coated with 6-μg extracts of a mixture of male and female worms, Mf, or ES of *B. malayi*, and Mf, or male, female, or a mixture of male and female worms of *B. pahangi* in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After removing excess antigen from the wells, the plates were blocked with 3% fish gelatin (Sigma) in carbonate buffer for 2 h at 37 °C. Immunized rabbit serum (100 μl of ab-A or ab-B) diluted 1:500 with PBS-Tween 20 (PBST) was added to each well of antigen-coated plates and incubated at 37 °C for 2 h. Normal rabbit serum was used as a control. Plates were washed with PBST, and then incubated for 2 h at 37 °C with antirabbit-IgG labeled with horseradish peroxidase (HRP; Kirkegaard and Perry Laboratories,

Gaithersburg, Md.). Plates were then washed with PBST; and a substrate solution consisting of ortho-phenylenediamine (OPD) and hydrogen peroxide (H_2O_2) was added to each well and incubated in the dark for 10–15 min at room temperature. The reaction was stopped with 1 N H_2SO_4; and A_{490} was measured with an ELISA reader (Dynatech Laboratories, Alexandria, Va.).

Alternately, GSTs were also detected by indirect-capture ELISA using polyclonal rabbit sera (ab-A and ab-B) to coat the ELISA plates, followed by incubation with *B. pahangi* adult worm extract at 10 μg ml^{-1}. Normal rabbit serum was used as a control. Washing with PBST was done at all steps before the addition of blocker (3% gelatin in carbonate buffer) and adult worm extract. The GST and antibody complex was determined by incubating the wells with diluted HRP-labeled antirabbit IgG followed by substrate addition. A_{490} was determined as described above. *B. pahangi* adult worm extract (10 μg) and the five affinity-purified fractions (5 μg) resolved on a 15% gel were electro-blotted onto a nitrocellulose membrane using standard methods. The blots were probed with ab-A or ab-B to detect the immunoreactivity of these sera using standard procedures (Bollag and Edelstein 1991).

Histological localization of GST in *B. pahangi* adult worms

For histological localization studies, *B. pahangi* adult male and female worms recovered from the peritoneal cavity of infected jirds were washed in PBS, fixed in ice-cold 10% PBS, processed in alcohol, and then fixed in paraffin. Longitudinal and transverse sections were made, fixed on clean glass slides, and processed for immunohistochemical analysis. The endogenous peroxidase activity was removed by 30 min incubation in 0.3% H_2O_2 in methanol at room temperature. The sections were blocked with 5% goat serum in PBST and then incubated for 2 h at room temperature with rabbit serum raised against glutathione-binding proteins (ab-A or ab-B). The serum was used at 1:50 dilution in PBST/1% goat serum. Pooled serum from normal rabbits was used as a control. After washing three times with PBS, the sections were incubated with biotinylated goat antirabbit IgG (Vector Labortatories, Burlingame, Calif.) diluted 1:50 in PBS/1% goat serum.

After three washes with PBST, the sections were incubated with HRP-labeled streptavidin (Vector Laboratories) for 45 min at room temperature. The sections were rinsed with PBST and then incubated with AEC chromogen for 10 min at room temperature. After rinsing three times with distilled water, the sections were counter-stained with Mayer's haematoxylin for 2 min at room temperature, mounted with an aqueous medium, and examined with an Olympus compound microscope.

Inhibitors

The following GST inhibitors were purchased from Sigma Chemical Co.: [2,3-dichloro-4-2-(2-methylenebutyryl]phenozyacetic acid, commonly known as ethacrynic acid (EA) or Edecrin (Merck, Whitehouse Station, N.J.), *N*-ethylmalemide (NEM), 4-nitropyridine-oxide (NPO), CDNB, oxidized glutathione (GSSG), and *s*-(*p*-azidophenylacyl)-glutathione; as were ascorbic, palmitic, and stearic fatty acids. Ascorbyl palmitate (Aldrich Chemical Co., Milwaukee, Wis.) and ascorbyl stearate (a gift from Hoffman La Roche, Basel, Switzerland) were also used. Phenoxyacetic acid (Sigma), the parent compound of EA (but lacking the active methylene adduct) and furosemide (Sigma), a diuretic compound functionally similar to EA, were tested for their ability to inhibit the viability of adult worms, Mf, and L3. Different concentrations of these inhibitors in soluble form were prepared fresh before they were added to medium. The pH of the working solution containing the inhibitor was adjusted to 7.5.

Effect of inhibitors on parasite viability

Adult male or female worms ($n = 2$) were incubated in 1 ml of either medium alone or medium containing the above inhibitors at appropriate concentrations in a 24-well culture plate (Costar, Cambridge, Mass.). Plates containing parasites were incubated at 37 °C in a 95% air/5% CO_2 atmosphere. Frozen parasites were thawed and used as negative controls. Experiments were carried out in duplicate, and the results were expressed as an average of two independent experimental values.

Assessment of parasite motility

Microscopic observation

Parasite motility and death were assessed visually using a Leitz inverted microscope, and the observations were scored as: −, immotile or dead; 1+, slightly active; 2+, active and motile; and 3+, highly active and motile, as rated against the activity and motility of worms in culture without inhibitors.

MTT reduction assay

Parasite viability was assessed quantitatively by the MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] reduction assay. The procedure used was as described by Rao et al. (1991). Briefly, after microscopic assessment of parasite viability, adult worms were transferred to 0.5 ml of PBS, containing 0.5 mg MTT ml^{-1} (Sigma). The L3 were suspended in 0.1 ml PBS containing 0.5 mg MTT ml^{-1}. Adult worms, Mf, or L3 were incubated for 2 h at 37 °C in the dark. Dimethylsulfoxide (DMSO, 100 μl) was added to dissolve the dark blue crystals of formazan; and the mixture was then transferred to a 96-well microtiter plate (Costar). Adult worms incubated in inhibitors were washed once with PBS and blotted; and each adult worm was then transferred into a well of a microtiter plate containing 200 μl DMSO. The parasite-DMSO mixture was incubated for 1 h at room temperature for complete solubilization of formazan from the parasites into the solvent. Formazan quantitation was performed with an automatic microtiter plate reader (Dynatech MR 5000) at a wavelength of 595 nm, using DMSO as a blank. Dead worms that had been frozen at −70 °C for several months were thawed and used as negative controls in this assay. These absorbance values, relative to those for the controls, were compared for differences in inhibition of viability. Greater than 50% inhibition in MTT reduction was arbitrarily considered significant. IC_{50} values for each inhibitor were obtained from a simple regression line plotted for percent inhibition versus inhibitor concentration.

Results

GST activity in soluble extracts of life cycle stages of *Brugia*

Colorimetric assay showed high levels of GST in somatic extracts of L3 (9 μmol min^{-1} mg^{-1} protein), L4 (3.9 μmol min^{-1} mg^{-1} protein), Mf (2.5 μmol min^{-1} mg^{-1} protein), male (1.7 μmol min^{-1} mg^{-1} protein), and female (0.9 μmol min^{-1} mg^{-1} protein). The enzyme activity was much higher in affinity-purified fractions of somatic extracts of adult worms. The first five eluted fractions (2 ml each) of adult worms were 940, 486, 270, 250, and 130 μmol min^{-1} mg^{-1} protein. These results confirm the previous observations of Jaffe and Lambert (1986) and indicate GST activity in the glutathione-binding proteins of adult *Brugia* worms. The cytosol fraction of jird liver, a positive control which was used separately in this colorimetric assay, showed

92.7 μmol min^{-1} mg^{-1} protein GST activity. This indicated that the enzyme assay we used detects GST profiles. Adult worm extracts of *B. malayi* and their ES products showed GST activity as measured in 1.5 and 1.02 μmol min^{-1} mg^{-1} protein, respectively. The lavage of intraperitoneally infected jirds with acute infections (90 DPI) showed enzyme activity that was three times higher than that seen in uninfected jirds (0.197 versus 0.064 μmol min^{-1} mg^{-1} protein).

Immunoreactivity of antisera to *Onchocerca volvulus* GSTs to *Brugia* extracts

In antibody-capture ELISA, 6 μg ml^{-1} of Mf, male, female, and mixed worm antigens of *B. pahangi* or Mf, adult worm, and ES antigens of *B. malayi* reacted strongly with rabbit antisera to *Onchocerca volvulus* glutathione-binding GSTs (Fig. 1A, B). These antigens also reacted strongly with antisera to ab-B and ab-A, compared with normal rabbit serum. Similarly, the absorbance values in antigen-capture ELISA indicated these two antibodies captured glutathione-binding GSTs of *B. malayi* adult worms (ab-A: $A = 0.260 \pm 0.008$; ab-B: $A = 0.452 \pm 0.10$; normal rabbit serum: $A = 0.08 \pm 0.02$), suggesting this family of proteins has a high degree of cross-reactivity. These results also demonstrate that the antisera reacted strongly with all types of parasite antigens. Glutathione-binding proteins of *B. pahangi* adult worms purified by affinity chromatography on a GSH-coupled-Sepharose 4B column showed three major bands with molecular sizes of 24, 32, and 36 kDa. These were predominantly seen in the first four fractions, with two proteins in the fifth fraction at 24 kDa and 32 kDa, suggesting these proteins have a high affinity for glutathione (data not shown). The results are comparable with the studies on *O. volvulus* proteins (Salinas et al. 1994) except that the 24/25 kDa doublet was not observed in our protein preparations. In Western blot experiments, 24 kDa *Brugia* protein was reacted with ab-A. However, *Brugia* proteins of 24 kDa and 32/36 kDa complex) reacted strongly with ab-B.

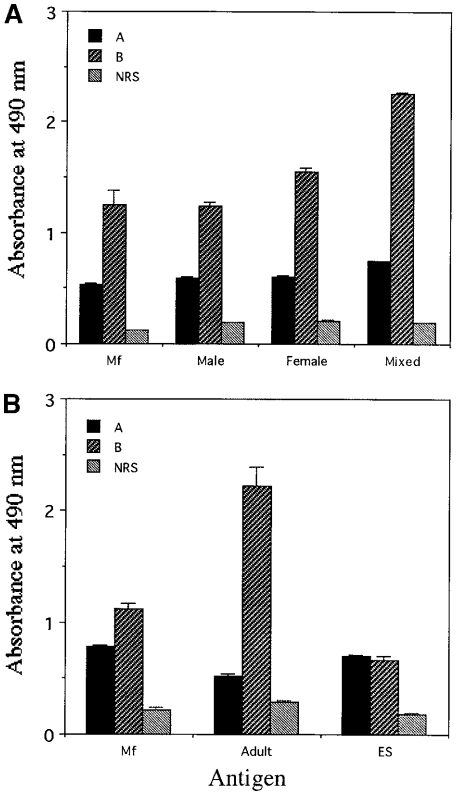

Fig. 1A, B Immunoreactivity of *Onchocerca volvulus* glutathione S-transferase antibodies to *Brugia pahangi* (*A*) and *B. malayi* (*B*) worm antigens (*Mf* microfilaria, *Male* male worms, *Female* female worms, *Mixed* mixed male and female worms, *Adult* adult male and female worms, *ES* excretory–secretory products of adult worms) as measured by enzyme-linked immunosorbent assay. Results are mean values ±1 SD. *NRS* Normal rabbit serum (control)

Immunohistochemical localization of GST

Because the antisera were ELISA-positive with *Brugia* antigens, we analyzed the sections of *B. pahangi* worms with ab-A and ab-B by immunohistochemical staining. These antibodies stained discrete areas of adult worms, whereas normal serum failed to show specific staining. Staining with ab-B was increased in the muscle layer and the hypodermis when compared with staining with ab-A (Fig. 2C, D). The fluid around the embryos and around the egg shells in the lumen of the uteri was stained strongly with ab-B (Fig. 2E). The Mf stages inside the uteri showed no increased staining for ab-A or ab-B compared with that seen with normal serum. In male worms, the somatic musculature and hypodermis were stained with antisera ab-B (Fig. 2F, G, H). Staining in musculature was seen with ab-A and ab-B (Fig. 2G, H). Specific staining was observed within the vas deferens of the testis (Fig. 2G, H) and did not resemble that seen in tissue sections treated with normal sera (not shown). Non-specific staining with ab-B was observed in the intestinal lining of male worms (Fig. 2F).

Effect of inhibitors on parasite motility and viability

To determine whether GSTs have any role in the motility and viability of *Brugia* spp., the effect of a battery of known GST inhibitors with excellent substrate specificity for mammalian GST was tested on the viability of female worms, male worms, L3, and Mf of *B. pahangi*. These stages were incubated separately in vitro with various inhibitors for 24 h and 48 h. The motility of parasites was determined microscopically and viability was measured by MTT reduction assay. At concentrations of 0.01–0.5 mM, GST inhibitors such as GSSG, *s*-(*p*-azidophenylacyl)-glutathione, ascorbic acid, stearic and palmitic fatty acids, and esters of ascorbic acid such as ascorbyl stearate or ascorbyl palmitate showed no

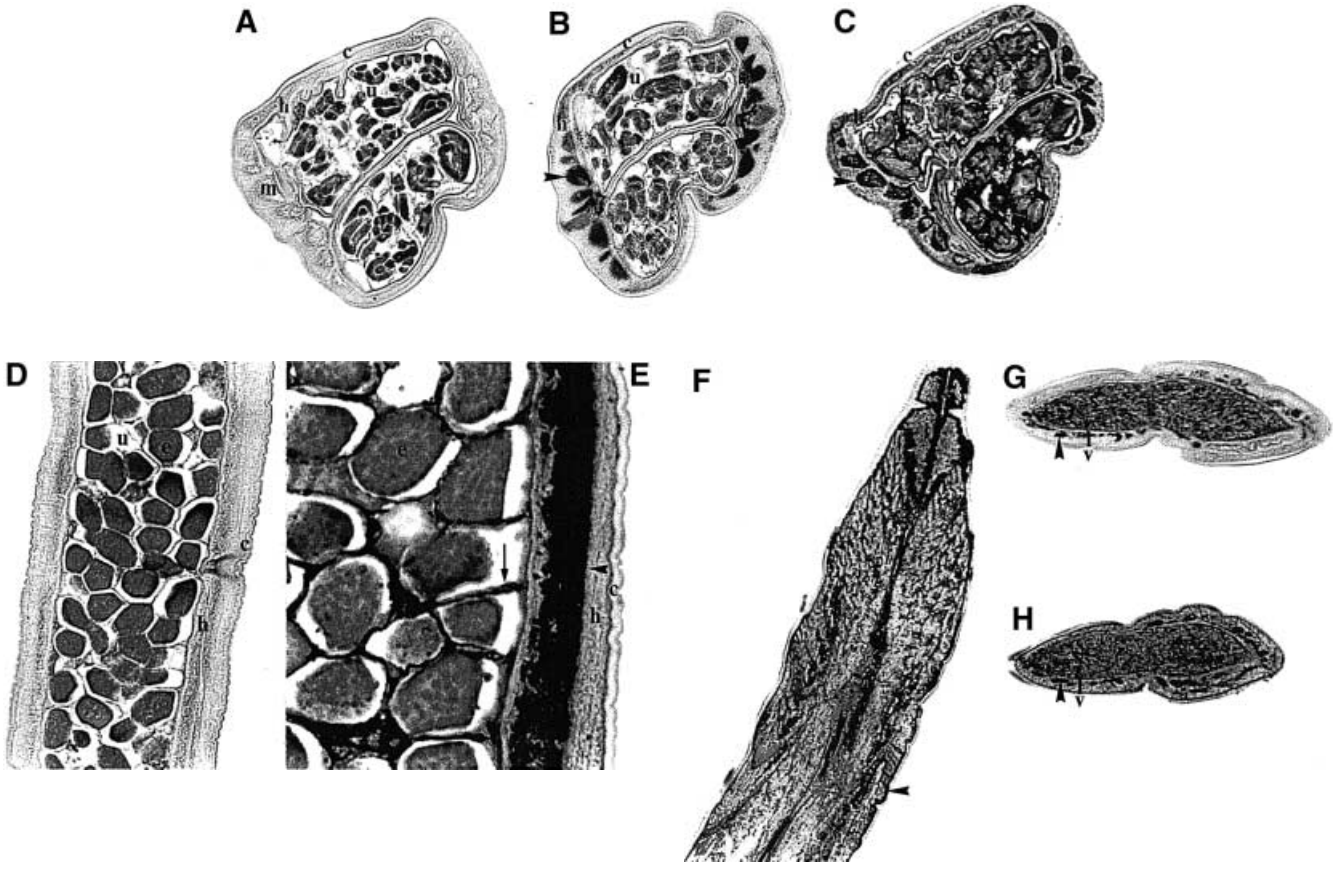

Fig. 2 Immunohistochemical localization of glutathione-binding proteins in female (**A–E**) and male worms (**F–H**) of *B. pahangi*. Paraffin sections (**A–C, G,** and **H** are cross sections, **D** and **E** are longitudinal sections, and **F** is an oblique section) were immunostained with rabbit antibodies to affinity-purified fractions of glutathione-binding proteins of *Onchocerca volvulus* (ab-A or ab-B). Sections were stained with normal rabbit serum (**A** and **D**), ab-A (**B** and **G**), or ab-B (**C, E, F,** and **H**). *c* Cuticle, *e* embryos, *h* hypodermis, *i* intestine, *m* muscle, *u* uterus, *v* vas deferens, *s* spermatozoa. ×360. *Arrow heads* indicate the location of muscle bands in hypodermis. *Arrow* indicates the staining of fluid in between the embryos

effect on parasitic motility or viability (data not shown). Likewise, similar concentrations of phenoxyacetic acid and furosemide did not affect the motility or viability of the parasite at any life cycle stage.

However, at equimolar concentrations, EA, NEM, NPO, and CDNB individually reduced the motility of adult worms, MF, and L3 by 24 h and reduced viability by 48 h. EA, NEM at 0.3 mM, and CDNB individually inhibited worm viability by 24 h (1+). The inhibition of motility with these inhibitors and NPO was significantly increased by 48 h at 0.05 mM, when worms appeared dead and immotile (–). The individual effect of EA, NEM, NPO, or CDNB on viability was further tested by determining the ability of parasites to reduce the tetrazolium salt MTT to its formazan product under conditions of an active respiratory metabolism. At 48 h, these inhibitors reduced formazan formation. Conversely, adult worms cultured without inhibitors actively incorporated MTT and reduced it to purple formazan. Viability of adult worms was decreased by 50% or more with these inhibitors at concentrations greater than 0.01 mM and above, compared with that of control worms (Fig. 3A, B). The viability of Mf and L3 was similarly reduced (Fig. 3C, D). The effect of CDNB on Mf viability was not tested in this assay. PA showed no effect on Mf viability. In summary, the addition of inhibitors to the cultures reduced the viability of female worms ($IC_{50} = 0.028$ mM EA, 0.018 mM NEM, 0.064 mM NPO, 0.016 mM CDNB), male worms ($IC_{50} = 0.052$ mM EA, 0.03 mM NEM, 0.015 mM NPO, 0.015 mM CDNB), Mf ($IC_{50} = 0.045$ mM EA, 0.042 mM NEM, 0.22 mM NPO), and L3 ($IC_{50} = 0.02$ mM EA, 0.015 mM NEM, 0.052 mM NPO, 0.015 mM CDNB). EA and CDNB also markedly reduced *B. malayi* adult female viability (data not shown). Frozen worms showed no effective incorporation of MTT and therefore formazan values were extremely low and were comparable to DMSO background values.

Discussion

To survive in a hostile environment, filarial nematodes have adopted a number of strategies to evade, modify or neutralize the hosts' defense mechanisms. Increasing evidence shows that a parasite's enzymatic pathways

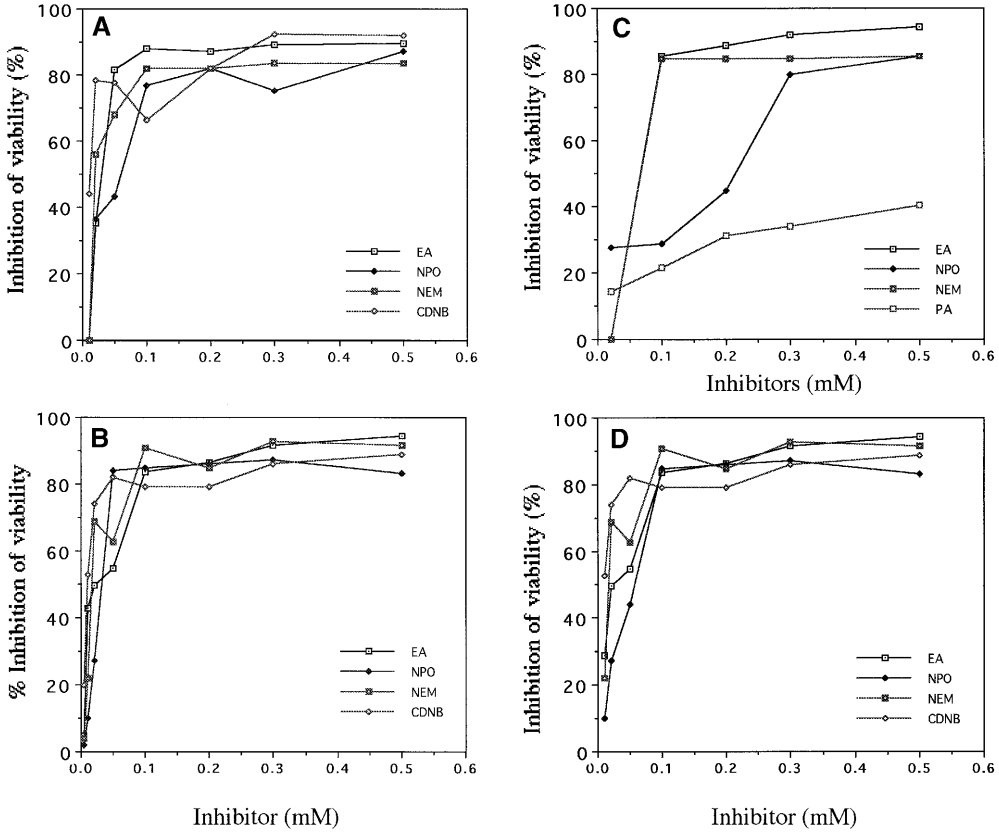

Fig. 3 Effect of inhibitors on the viability of *B. pahangi* adult female (**A**), and male worms (**B**), microfilariae (**C**), and third stage larvae (**D**). Values are the mean of duplicate samples of two independent experiments. The standard deviation between duplicate samples never exceeded 10% and did not vary with concentration. The percentage of viability inhibition was calculated from MTT reduction assay values of control worms incubated without inhibitors. Decrease in viability showing more than 50% was considered effective. *EA* Ethacrynic acid, *NEM* *N*-ethylmalemide, *NPO* 4-nitropyridine-oxide, *CDNB* 1-chloro-2,4-dinitrobenzene, *PA* phenoxyacetic acid

play an important role in effectively orchestrating these strategies (James 1994; Selkirk et al. 1998). GST activity in relation to these strategies has been indentified in filarial nematodes and other helminths (Brophy and Pritchard 1994). Although isozymes of filarial-GST homologs have been cloned and expressed (Liebau et al. 1994b, c; Salinas et al. 1994), the effectiveness of GSTs as molecular targets for the development of antifilarial drugs and their use in recombinant forms to induce protective immunity remain to be determined. We studied GST activity in the life cycle stages of *Brugia* spp. Our results obtained with adult worm extracts and affinity-purified fractions agree with those previously reported (Jaffe and Lambert 1986). The high GST activity we observed in L3, L4, and ES products, compared with that in adult worms, suggests that this enzyme is ubiquitous in all stages of the parasite's life cycle. The GST activity we observed in the peritoneal lavage of infected jirds indicates that GST may be secreted into the peritoneal cavity. We observed a marginal increase in the GST activity of peritoneal exudate proteins of infected jirds, compared with that of uninfected jirds. This could be due to the competition of respective proteins in our enzyme assay. Additional studies are needed to further characterize the secretory nature of worm GSTs, by using affinity-purified fractions of these proteins in enzymatic assays. The elevated levels of GST protein expression in L3 and L4 may be involved in the parasite's strategy for evasion, aimed at a host's early defense mechanisms and may also assist in larval migration to the site of predilection. Native and the secretory GSTs may play a similar role in adult worm survival. L3 and L4 ES products may also contain GSTs, but this remains to be determined.

Our immunoreactivity results indicate that antisera of *Onchocerca* GSTs cross-react with *Brugia* proteins and effectively bind to *Brugia* antigens, suggesting that they are possibly the same family of proteins. In this study, lack of purified *Brugia* GSTs precluded its further use in developing *Brugia* GST antisera in rabbits. However, highly cross-reactive *Onchocerca* GST sera with *Brugia* GST was useful for extending our studies. Immunohistochemical results showed that antisera raised against a mixture of glutathione-binding GSTs of *O. volvulus* have increased reactivity over antisera raised against a single protein. Both however were observed mainly in the worm hypodermis, which is important in the passive transport of molecules between the cuticle and the environment, and in the muscle bands, where high metabolic activities occur. Glutathione-binding GSTs were also predominantly located in the reproductive organs of adult worms. In adult female worms, strong reactivity was noted in the uterine contents, indicating that embryos are particularly susceptible to oxidative stress. Interestingly, microfilariae within the uterus did not increase the reactivity with immune sera and the staining pattern was similar to normal serum.

In adult male worms, specific staining was observed in hypodermis, muscle and vas deferens containing spermatocytes. Previous immunohistochemical studies

localized these proteins in the hypodermis, the wall of the seminal receptacle, and the spermatozoa of adult worms of *O. volvulus* (Salinas et al. 1994). Using antisera to recombinant OvGST1, Liebau et al. (1994b) observed staining in the outer cellular covering of the worm and the syncytial hypodermis. GSTs have also been found in sperm cells in *O. volvulus* (Salinas et al. 1994) and superoxide dismutase has been identified in the seminal fluid of *B. malayi* (Ou et al. 1995). Molecular characterization and ultrastructural localization studies with two forms of glutathione S-transferases indicate striking differences between a 31 kDa OvGST1 which can be secreted out of the hypodermis into the cuticle and a 24/25 kDa OvGST2 which is an intracellular cytosolic protein and functions as a house-keeping enzyme (Wildenburg et al. 1998). The 24 kDa OvGST2 was shown to have similarities with Pi class GSTs, which differ from OvGST1 biochemically and in tissue distribution (Liebau et al. 1996). All these studies indicate that glutathione-binding proteins in worms are predominantly associated with metabolic and reproductive sites. The identification of a specific rat GST isozyme that is abundant in spermatogenic tubules and exhibits high GSH-peroxidase activity with pyrimidine hydroperoxides as substrates suggests that GSTs may be important to the fidelity of DNA replication by detoxifying pyrimidine hydroperoxides (Tan et al. 1986).

The in vitro and in vivo effects of GST inhibitors on worms have not yet been reported. Of the known GST inhibitors we studied, EA, NEM, NPO, and CDNB reduced the viability and motility of parasites in vitro. GSSG, *s*-(*p*-azidophenylacyl)-glutathione, ascorbic, stearic and palmitic acids or ascorbyl esters, which have been shown to inhibit GST isozymes (Mitra et al. 1991), were inactive on GSTs at the concentrations we tested, indicating that GSTs have specific drug receptor interactions that may be different from those of mammalian systems. Recent evidence suggests that GST inhibitors may have important clinical implications (Kew et al. 1988; Hansson et al. 1991). Pi class GST has been detected in rapidly growing tumor cells (Sato 1989; Shea et al. 1988). Studies have also shown that GSTs may have an important role in tumor cell resistance to anticancer drugs. One can speculate from these studies that one or more of the GST isozymes may be involved in inactivating several anthelminthic drugs. EA, a phenoxyacetic acid derivative with a ketone and methylene group and a diuretic drug, has been shown to inhibit GST activity in a mammalian system (Ahokas et al. 1985; Ploemen et al. 1990) and, because it can markedly reduce worm viability, compounds in this chemical series may have therapeutic use. However its parent compound, phenoxyacetic acid, lacks the methylene adduct and both it and furosemide, a diuretic drug that is functionally similar to EA but is not a GST inhibitor, failed to show any inhibitory effect on parasitic worm viability or motility. This suggests that the inhibitory effect of EA on viability is not due to its diuretic function. Although the effect of EA in individuals infected with filarial worms is unknown, because it is a potent diuretic agent, it is not safe to use for prolonged periods of time. Therefore, further search is needed for relatively non-toxic GST inhibitors as antifilarial agents. Knowledge of the gene structure of both the mammalian GST (Pickett and Lu 1989) and the filarial GST (Salinas et al. 1994; Libeau et al. 1996; Rathur and Libeau 1997) and active site configuration will enable the synthesis of specific inhibitors with which GST biochemical pathways can be targeted. In addition, with the availability of recombinant GST isozyme proteins, prototype vaccine formulations may be tested against filarial infections.

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