Differences in Sensitivity of *Schistosoma mansoni* Schistosomula, *Dirofilaria immitis* Microfilariae, and *Nematospiroides dubius* Third-Stage Larvae to Damage by the Polyamine Oxidase-Polyamine System

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The effect of the polyamine oxidase (PAO)-polyamine system on some helminths was examined in vitro. Both *Schistosoma mansoni* schistosomula and *Dirofilaria immitis* microfilariae were highly sensitive to this system, the latter more so than the former. In contrast, exsheathed third-stage larvae of *Nematospiroides dubius* were resistant to the effects of the PAO-polyamine system. After incubation of microfilariae with either spermine or spermidine in the presence of serum containing PAO (bovine serum or human retroplacental serum) or partially purified PAO, damage of worms occurred, compatible with our criteria for worm death. Similar results were obtained with schistosomula by using spermine. The damage seemed to be mediated by PAO products other than hydrogen peroxide because catalase did not protect either parasite. Our data demonstrate that helminths may be damaged by products of the PAO-polyamine system.

Polyamine oxidases (PAOs) have been found in most mammalian tissues (15, 24). Studies in the past have concentrated mainly on PAOs from two sources, bovine serum and rat liver (13–15). PAO from bovine serum acts on the primary amino groups of spermine to form an aminodialdehyde, while rat liver PAO cleaves spermine at secondary amino groups, with the formation of spermidine and 3aminopropionaldehyde (13; Fig. 1). In both reactions, hydrogen peroxide (H₂O₂) is formed. Preliminary studies have also been conducted with macrophage and human pregnancy serum PAOs. These resemble the rat liver enzyme (14, 15, 18; Fig. 1).

Recently it was shown that trypanosomes are rapidly killed when incubated with PAO or sera containing PAO and polyamines (6, 7). Although this system was postulated to be of special relevance as a nonspecific trypanocidal mechanism operating in ruminants (6), it may be of more general importance since PAOs may be elevated during some infections in nonruminants and during human pregnancy (13, 15, 20). Also, macrophages, when activated, have been shown to contain higher quantities of PAOs and appear to secrete the enzyme(s) (20).

In view of these findings, it was of interest to determine the susceptibility of helminths to the products generated during the oxidation of polyamines by both the bovine serum and human pregnancy serum PAOs. We studied *Schistosoma mansoni* schistosomula, *Dirofilaria immitis* microfilariae, and exsheathed third-stage larvae of *Nematospiroides dubius*.

**MATERIALS AND METHODS**

**Parasites.** Microfilariae of *D. immitis* were prepared from freshly collected, heparinized dog blood by filtration through polycarbonate filters (pore size, 5 μm) and were used after overnight incubation at 37°C (22). More than 95% of microfilariae showed normal motility after recovery by this procedure.

*S. mansoni* Puerto Rican strain was maintained by passage in laboratory-bred albino *Biomphalaria glabrata* snails and hamsters essentially as described previously (25). The schistosomula used were prepared from cercariae by mechanical transformation (4, 10).

Exsheathed third-stage larvae of *N. dubius* were kindly provided by Charles R. Jenkin, Department of Microbiology and Immunology, University of Adelaide.

**Sera.** Bovine serum was obtained from 9- to 14-month-old cows. Human retroplacental serum (RPS), which was composed mainly of inter villous blood and included some decidual and placental interstitial fluid, was prepared as previously described (19). All sera were heated to 56°C for 20 min and stored in portions at −70°C.

**Enzymes.** PAO (bovine plasma, EC 1.4.3.6) was obtained from Miles Laboratories, Goodwood, South Africa. This is described as amine:oxygen oxireductase (deaminating) with a specific activity of 23.55 U/g of protein, where 1 U is the amount of enzyme required to form 1 μmol of benzylaldehyde per min from benzylamine at 25°C. Catalase (bovine liver, EC 1.11.1.6) was purchased from Sigma Chemical Co., St. Louis, Mo.

**[¹⁴C]spermine oxidation assay.** PAO activity was measured by a radiochemical method (8, 20). To a 0.1 ml of a 1,000-μg/ml mixture of purified beef plasma PAO was added 0.1 ml of medium substrate solution (radioactive spermine, 0.415 μCi/ml plus 5 mM cold spermine [1.17 μg/ml]), 0.05 ml of human serum (added as carrier protein with no effect on activity), and 0.05 ml of medium. These were incubated at 37°C for 90 min, and then 0.05 ml of 50% (wt/vol) trichloroacetic acid was added to stop the reaction. The protein was sedimented by centrifugation at 400 × g for 10 min, and the products in the supernatant were separated from unreacted spermine by column chromatography on a strong cation exchange resin (Dowex-50W-X2 resin).
Spermine and reaction products were eluted with a stepwise gradient of hydrochloric acid, and the percent substrate converted was calculated. The enzyme activity was expressed in international units (micromoles of product formed per minute). The activity in RPS was similarly assayed by using 0.1 ml of RPS in the assay. The PAO activity was 33,000 mU/g in the commercial PAO preparation material and 5,000 mU/liter in the RPS.

Reagents. Spermine tetrahydrochloride and spermine trihydrochloride were obtained from Sigma Chemical Co.; [14C]spermine (N′N′-bis[3-aminopropyl]-1,4-tetramethylene-1,4-diamine trihydrochloride) was from Amersham Corp., Amersham, England.

Experimental assays. Experiments were set up in flat-bottomed microtiter plates (Linbro; Flow Laboratories, Inc., McLean, Va.). To 50 μl of the worm suspension containing either 100 microfilariae, 100 N. dubius larvae, or 50 schistosomula was added 50 μl of the PAO-containing serum or purified PAO, to the concentrations indicated in Results and Table 1, and 100 μl of a polyamine concentration. Controls were set up in which worms were incubated with one of the following: PAO preparation or serum, polyamines in human serum albumin, or albumin only (the use of bovine serum albumin was avoided since this may be contaminated with PAO). The plates were incubated at 37°C for 18 h (unless otherwise specified) in an atmosphere of 5% CO₂ and high humidity. At the indicated times the organisms were examined with an inverted microscope under phase at ×200 magnification. Death was scored as follows: for schistosomula, loss of motility, obvious tegument deformations, and granular appearance were the main criteria; for microfilariae and N. dubius larva, loss of motility was the main criterion indicating death (organisms which were immobile during the period of scoring were considered dead).

Antibody to detect binding of mouse antibodies to schistosomula antigens.

RESULTS

Effects of bovine serum and polyamines. Examinations of microfilariae and schistosomula after incubation with 10% bovine serum and 100 μM spermine for 18 h indicated that these conditions resulted in worm damage. Most schistosomula lost motility and showed morphological changes consistent with severe structural damage and schistosomula death. The surface of the tegument exhibited deformations and elaborations, and some of the worms showed extrusion of their content. All the microfilariae lost motility and straightened. Microfilariae and schistosomula incubated in either bovine serum or spermine alone did not display the effects described above and retained normal motility and appearances.

Effects of partially purified PAO. Microfilariae and schistosomula were incubated in the presence of 100 μg of PAO per ml and a range of concentrations of spermine or spermidine for microfilariae and spermine for schistosomula. The PAO, in the presence of polyamines, mediated damage in schistosomula and microfilariae (Fig. 2, 3). The minimal concentrations of spermine and spermidine producing damage in the majority of the microfilariae were approximately 5 and 10 μM, respectively (Table 1). The minimal concentration of spermine required to produce similar levels of damage in schistosomula was approximately 100 μM (Table 1). The PAO alone had no effect on the worms. The spermine and spermidine dose-related effects are presented for microfilaria in Fig. 4, and the PAO dose-related effect is presented in Fig. 5.

Time to effect damage was dependent on the concentrations of PAO and polyamines. In the presence of 50 μg of PAO per ml and 25 μM spermine, 90% of microfilariae were immobilized after 90 min. In the presence of 100 μg of PAO...

FIG. 1. Action of bovine plasma PAO (top) and rat liver or RPS PAO (bottom) on spermine and spermidine.
per ml and 200 μM spermine. >90% of schistosomula were damaged after 120 min.

Preincubation of 100 μg of PAO per ml and 100 μM spermine for 1 h and subsequent heat inactivation of the enzyme (65°C for 1 h) did not decrease the capacity of this system to damage either microfilariae or schistosomula. In contrast PAO heated (65°C for 1 h) before spermine addition had no effect.

Larvae of N. dubius were insensitive to the PAO-polyamine system. Incubation in the presence of 1 mM spermine and 1 mg of PAO per ml for 3 to 4 days had no effect on the motility of these worms.

An attempt was made to assess the damaging effect of the PAO-polyamine system in relation to the ability of schistosomula to develop into adult worms in mice. Schistosomula were incubated in vitro with 100 μg of PAO per ml and 200 μM spermine for 18 h and then injected intravenously into five mice. Other groups of mice were injected with PAO- or spermine-treated schistosomula. After 8 weeks mice were perfused for adult worm recovery. Serum was also collected from these mice. No adult worms were recovered from mice receiving schistosomula exposed to PAO plus spermine. Antibodies to tegument and somatic antigens but not to gut-associated antigens were present in sera from these mice. Adult worms were recovered (12 to 16% recovery) from mice receiving schistosomula treated

### Table 1. Effect of partially purified PAO and polyamines and of RPS and polyamines on microfilariae and schistosomula

<table>
<thead>
<tr>
<th>Parasite treatment</th>
<th>Minimal polyamine concn (μM) required to damagea</th>
<th>Microfilariae</th>
<th>Schistosomula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partially purified PAO (100 μg/ml)</td>
<td>Spermine 5, 5, 2.5, 200, 200, 100, 100, 100</td>
<td>10, 10, 5</td>
<td>NDb</td>
</tr>
<tr>
<td>RPS (10%)</td>
<td>Spermine 62.5, 62.5, 31.25, 200, 100, 100</td>
<td>125, 125, 62.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Damage criteria: microfilariae, >80% demonstrating immobility; schistosomula, >80% demonstrating immobility, opaque granular appearance, and membrane deformations. Values represent determinations made in separate experimental runs. The incubation time was 18 h.

b ND, Not done.

![Photomicrograph showing the typical appearance of microfilariae treated with PAO and spermine (A) and spermine only (B). The parasites lacked motility, straightened, and shrank after treatment with PAO and spermine. This contrasted with the appearance of microfilariae in control cultures, e.g., treated with spermine only, in which the parasites retained normal motility similar to that exhibited by worms freshly prepared from dog blood.](http://iai.asm.org/)

![Effect of different concentrations of spermine and spermidine on the antimicrofilarial action of the PAO-polyamine reaction. Symbols: ● and ■, spermine and spermidine, respectively, in the presence of 100 μg of PAO per ml; ○, spermine alone; □, spermidine alone. Examination of cultures was made after 18 h of incubation, and results are expressed as the means plus or minus standard deviations of five experiments for spermine and of three experiments for spermidine.](http://iai.asm.org/)

![Effect of different concentrations of PAO on the antimicrofilarial action of the PAO-polyamine reaction. Cultures contained PAO and 50 μM spermine. Examination of cultures was made after 18 h of incubation, and results are expressed as means plus or minus standard deviations of three experiments.](http://iai.asm.org/)
with PAO only, and sera from these animals contained antibodies to tegument, somatic, and gut-associated antigens. Although no adult worms were recovered from mice treated with spermine alone, there was evidence of an infection based on the serology results which showed the presence of antibodies to gut-associated antigens.

**Effects of catalase on PAO-mediated worm damage.** PAO (40 μg/ml) and 10 μM spermine were preincubated in the presence or absence of 1,000 U of catalase per ml for 15 min before the addition of microfilariae. Worm damage was scored after 18 h of incubation. Catalase was unable to abolish the PAO-mediated damage of microfilariae, indicating that the effects were not due to hydrogen peroxide (data not shown). Similar results were obtained with schistosomula by using 100 μg of PAO per ml and 200 μM spermine. This result was obtained in four experiments.

**Effect of acrolein.** Microfilariae were incubated with a range of concentrations of acrolein, and worm damage was scored after 2 h of incubation. A typical experimental run showed that 25 μM acrolein or 5 μM spermine (the latter in the presence of excess PAO) was required to achieve damage in at least 90% of worms.

**Effect of RPS and polyamines.** Microfilariae or schistosomula were incubated with 10% RPS and a range of concentrations of polyamines. Both were damaged when incubated in the presence of RPS and polyamines. The appearance of the worms was similar to that after their incubation in bovine PAO and polyamines. The minimal concentrations of spermine and spermidine producing damage of the majority of the microfilariae were approximately 60 and 125 μM, respectively (Table 1). The minimal concentration of spermine to achieve marked damage to the majority of the schistosomula was 100 μM (Table 1). A polyamine dose-related effect on microfilariae is presented in Fig. 6. This effect was not observed with either RPS or polyamines alone (i.e., in human AB serum).

**DISCUSSION**

Previously, evidence was presented showing that protozoan parasites of the genera *Plasmodium* and *Trypanosoma*, in addition to tumor cells (1), are highly sensitive to products of the PAO-polyamine reaction (6–8, 16, 17, 23). We now present evidence that some helminth parasites were also highly sensitive to this system. *Dirofilaria immitis* microfilariae were immobilized, relatively small concentrations of spermine or spermidine in the presence of either PAO-containing bovine serum or a partially purified PAO preparation. Similarly, schistosomula were markedly damaged when incubated with spermine and either bovine serum or PAO. However, exsheathed third-stage larvae of *N. dubius* were resistant to the damaging effects of the PAO-polyamine system. The killing of the parasites appeared to be a result of extracellular generated toxic products rather than an effect caused by the uptake of PAO and spermine by the parasites. Evidence showed that preincubation of PAO and spermine and subsequent heat inactivation of PAO did not decrease the ability of PAO to kill the worms.

A number of products formed during the oxidation of spermine were shown to be toxic to microorganisms and tumor cells. These included H₂O₂, spermidialdehyde or 3-aminopropionaldehyde, and acrolein. H₂O₂ is a product common to both bovine PAO- and RPS-polyamine-mediated polyamine oxidation. While microfilariae are highly sensitive to H₂O₂ concentrations expected to be generated in the PAO-polyamine reaction (22), the inability of catalase to prevent damage suggests that other products besides H₂O₂ are responsible for the effects of PAO-polyamine reaction. Acrolein, a breakdown product of the primary products, aminodialdehydes and aminoaldehydes, was found to be toxic for microfilariae, and under conditions similar to those in the experiments, acrolein could be detected during bovine PAO-polyamine reaction (8). However, it is unlikely that acrolein plays a significant part in the killing mediated by PAO-polyamines for two reasons. First, we found that 5 times as much acrolein was required to get the same level of damage as that expected to be generated at the lowest concentrations of polyamines causing >90% of worms to be damaged. Second, the aminoaldehydes were highly reactive, and their reaction with microfilariae would greatly limit the concentration of acrolein generated.

H₂O₂ is unlikely to be the main product responsible for schistosomula damage for two reasons. First, we were unable to prevent damage of schistosomula in the PAO-polyamine system by the addition of catalase. Second, it has been reported that schistosomula are not sensitive to H₂O₂ (11). Superoxide does not appear to be detectable during the reaction of polyamines with PAO (15). Thus, we have not considered this oxygen metabolite in the mechanisms of PAO-mediated worm damage.

Ferrante et al. (8) demonstrated that human RPS PAO, when reacted with polyamines (spermine or spermidine), generates trypanocidal products. Both microfilariae and schistosomula were sensitive to the RPS PAO-polyamine system. Studies with *Babesia* and *Plasmodium* species have shown that, although these parasites are susceptible to the action of bovine serum PAO, the enzyme present in RPS appears not to be able to mediate killing of intracellular hematoprotozoa (16, 17) including *P. falciparum* (C. M. Rzepczyk and A. Ferrante, unpublished data). The present results with RPS thus suggest a more general relevance of the PAO-polyamine system.

Since it is believed that the human serum (RPS) PAO...
resembles the macrophage PAO (14, 15, 18), macrophages may be involved in the damage and killing of some microorganisms by utilizing the PAO-polyamine system. Morgan and Christensen (16) have shown that spleens of mice infected with B. microti contained a threefold increase in PAO specific activity. In addition, the spleen size was as much as fourfold that of normal mice, showing a marked increase in total enzyme activity per spleen. Macrophages exposed in vitro to lipopolysaccharide appear to secrete PAO (18). These preliminary studies suggest that, upon activation, the macrophage may have increased quantities, if not increased activity, of PAO. However, it remains to be explored whether these changes significantly contribute to macrophage-mediated killing mechanisms.

Macrophages in cooperation with immunoglobulin E immune complexes mediate schistosomula cytotoxicity (3). In addition, other studies have shown that macrophages, activated by injecting mice with Mycobacterium bovis BCG or Corynebacterium parvum, killed schistosomula (12). Similarly, lymphokine-activated macrophages developed anti-schistosomula activity (2). The mechanisms by which activated macrophages mediate killing of schistosomula are still under investigation. Our results suggest that the PAO-polyamine system could contribute to schistosomula killing by activated macrophages. However, the significance of our finding in relation to PAO-polyamine killing of D. immitis microfilariae is less clear since the principal effector cell involved in the killing of this parasite (5, 22) is the neutrophil and we have no information on whether PAO is present in this cell type.

There was clear evidence from our study that, under in vitro conditions, microfilariae of D. immitis and schistosomula of S. mansoni are damaged by the PAO-polyamine system. Although there was some difficulty in interpreting the effects of this system on the ability of schistosomula to develop into adult worms, serological evidence did suggest that PAO-spermine-treated parasites, but not those treated with spermine or PAO alone, developed into adults. It is plausible to envisage an in vivo relevance for this system. First, tissue fluids of some animals (ruminants) contain naturally occurring high levels of PAO activity. In other animals the levels may rise during an infection, such as the increases that occur in spleens of Babesia-infected mice or in sera of some patients with hepatitis (13, 16). Second, polyamines are present in all living tissues, and their concentrations in tissue fluids rise dramatically as a consequence of tissue damage and regeneration (9). Parasites can themselves be a source of polyamines. Indeed, it has been recently reported in relation to African trypanosomiasis infections that resistant cattle have significantly higher levels of PAO than susceptible breeds (21). However, any in vivo role for this system should be cautiously extrapolated since the levels of enzyme and substrate as well as the existence of optimal conditions for the oxidation reaction at sites of parasite microenvironments are not known.

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LITERATURE CITED