Enhancement of *Schistosoma mansoni* Infectivity by Intradermal Injections of Larval Extracts: A Putative Role for Larval Proteases

Padraic G. Fallon, Mauro M. Teixeira, Carl M. Neice, Timothy J. Williams, Paul G. Hellewell, and Michael J. Doenhoff

Extracts of *Schistosoma mansoni* cercariae caused increased vascular permeability and edema if administered to CBA/Ca mice by intradermal injection. Percutaneous infection with cercariae over the skin site at which cercarial homogenate (CH) had been injected intradermally resulted in a significant increase in the infectivity of *S. mansoni* compared with that shown by worm recovery from control animals ($P < .05$). This effect was abrogated by inhibition of protease activity prior to injection. Injection of inflammatory mediators (bradykinin or zymosan-activated plasma) with or without prostaglandin E$_2$ produced a similar amount of edema as did CH. Injection of these mediators did not, however, enhance infectivity of cercariae. Pancreatic elastase was found to induce edema and enhancement of infectivity comparable to those induced by CH. The proteases introduced into the site of infection may have facilitated larval migration directly by hydrolyzing host tissue or indirectly by inducing an inflammatory response (or both).

Schistosomiasis is a parasitic disease of the tropics and subtropics, with >250 million people estimated to be infected [1], the majority with *Schistosoma mansoni*. The parasite is a trematode, the cercariae of which penetrate the skin of the primary host and eventually gain access to the systemic circulation. Penetration is believed to be achieved via a combination of mechanical and proteolytic means [2–4]. Relevant proteases are synthesized during cercarial development in the intermediate snail host and stored in the acetabular glands of the cercariae, from which they are released during penetration of host skin. Cercariae can be induced in vitro to secrete material from the acetabular glands by mechanical or skin lipid-induced transformation into schistosomula [5]. The secretions are highly proteolytic, with major proteolytic enzymes of 28–30 and 60 kDa [6, 7] and a more recently described activity at 47 kDa [8].

Extravascular leakage of plasma proteins occurs at the site of penetration by cercariae into the skin of experimental animals [9]. We recently investigated mechanisms of local inflammatory responses that occur after an intradermal (id) injection of cercarial extracts into guinea pig skin [10]. There was a strong correlation between the amount of proteolytic activity in cercarial extracts and their ability to induce both extravasation of plasma proteins and accumulation of eosinophils and neutrophils at the site of injection. Edema formation was dependent on local production of bradykinin [10]. The significance of this inflammation is unknown; it was suggested that it could be beneficial to either the parasite or the host [10].

This study is an investigation into the inflammatory and infection-enhancing properties of cercarial extracts when injected into mouse skin, with particular emphasis on a possible role for larval proteases in mediating these effects. The consequences of id administration of the known inflammatory mediators bradykinin (Bk), prostaglandin E$_2$ (PGE$_2$), and zymosan-activated plasma (ZAP) and of a purified nonparasitic protease on edema formation and subsequent *S. mansoni* infection were also evaluated.

**Materials and Methods**

*Parasite and laboratory host.* A Puerto Rican strain of *S. mansoni*, routinely maintained in laboratory passage in random-bred Tyler’s Original mice and albino * Biomphalaria glabrata* snails, was used. Age- and sex-matched CBA/Ca mice (20–35 g) were used for all experiments.

*Preparation of cercarial extracts.* *B. glabrata* snails with patent *S. mansoni* infections were induced to shed cercariae by exposing ~50 snails in 100 mL of water at 31°C to a strong light source for 2 h. Excess water was removed by filtration, and the larvae were sedimented by incubation of 10-mL volumes, each containing ~10$^5$ organisms, at 4°C. Supernatants were removed and the larval pellets stored at −20°C. The cercariae-free supernatants (snail water) and water in which the snails were maintained (pond water) were collected for analysis of the potential effects of possible bacterial or fungal contaminants on mouse skin. To avoid any osmotic-related effects, the pond and snail water were lyophilized and reconstituted with isotonic saline.

Cercarial transformation fluid (CTF) and cercarial homogenate (CH) were produced by a slight modification of the method described by Teixeira et al. [10]. Briefly, CH was generated by sonication of thawed larval pellets prepared as described above,
with \(3 \times 10^6\) cercariae resuspended in 4 mL of isotonic saline. After sonic disruption, the solution was centrifuged at 12,000 g for 10 min at room temperature to remove particulate debris. The supernatants had a protein content of 1.5–2.0 mg/mL and were stored at \(-20^\circ\text{C}\).

CTF was prepared by resuspending sedimented live cercariae in medium 199 containing 1% penstreptavidin (1.5 \(\times\) 10⁶ larvae/10 mL of medium) and aspirating this product 20 times through a 0.8 \(\times\) 40 mm needle. The larval suspension was then incubated for 2 h at 37°C, after which it was centrifuged and the supernatant stored at \(-20^\circ\text{C}\). Concentrated CTF (cCTF) was produced from CTF by ultrafiltration over a YM2 filter (Amicon, Beverly, MA) to yield a solution containing \(\approx 800\ \mu\text{g}\) of protein/mL.

Schistosomulum homogenate (SH) was obtained from the larval pellet after centrifugation and collection of CTF. The pellet was sonicated and centrifuged at 12,000 g for 10 min. The supernatant was removed and stored at \(-20^\circ\text{C}\). SH had a protein concentration similar to that of CH (1.5–2.0 mg/mL).

**Measurement of local edema formation in mouse skin sites.** 125I-labeled human serum albumin was injected intravenously into mice anesthetized with Hypnorm (Janssen Pharmaceuticals, Oxford, UK). After 10 min, cercarial extracts, commercial pancreatic elastase (E-1250, Sigma, Poole, UK), or solutions of known inflammatory mediators (Bk, ZAP [as a source of mouse C5a des-ARG], and PGE2) were prepared as described [10, 11]. Each animal received up to 6 injections administered in a random fashion, and the experiment was repeated on a total of 6 animals. After 1 h, animals were given a lethal dose of sodium pentobarbitone, and blood was taken by cardiac puncture. The injected skin sites were punched out and tested in a gamma counter together with plasma samples. Local edema formation was expressed as the ratio of [125I] counts in the skin sample divided by [125I] counts in 1 \(\mu\text{L}\) of plasma. Statistical differences between groups were assessed by one-way analysis of variance (ANOVA). \(P\) values were determined by the Student–Newman-Keuls post-hoc test.

**Azocoll assay.** Proteolytic activity of larval extracts and pancreatic elastase was determined by release of azo dye from collagen fibers using azocoll (Sigma) as described [10].

**Injection and infection of mice and retrieval of adult worms.** After they had been anesthetized with sodium pentobarbitone, mice were infected percutaneously with 200 \(S.\ mansoni\) cercariae. Adult worms were recovered by portal perfusion 6 weeks after infection, as previously described [12, 13]. Larval extracts (50-\(\mu\text{L}\) volumes adjusted to contain equivalent protein concentrations) were injected intradermally on the shaved abdomens of animals immediately after they had succumbed to the anesthetic. Within 5 min of the id injection, a suspension of 200 cercariae in 750 \(\mu\text{L}\) of water was placed within a plastic ring (1 cm in diameter) that had been centrally located over the injection site. In each experiment, all intradermally injected and noninjected control animals were infected at the same time. Worm recoveries from experimental groups (± 1 SE) were determined. ANOVA was used to test for statistical differences between experimental groups; \(P < .05\) was considered significant. A Dunnett test identified statistically distinct data sets. All infection studies were done twice.

**Results**

**Local edema caused by cercarial extracts in the skin of CBA/Ca mice.** An id injection of CH, SH, or cCTF, either undiluted or diluted 10-fold with saline, induced significant dose-dependent edema formation in mouse skin (figure 1). CH and SH were of a similar efficacy and some 3-fold more effective than cCTF. Preliminary experiments established that 10 mM PMSF caused complete inhibition of proteolysis by CH, as judged by azocoll degradation (data not shown). A concentration of 1.25 mM phenylmethylsulfonyl fluoride (PMSF), which inhibited azocollagenolytic activity of CH by 79%, was used for the following experiment, as higher PMSF concentrations had intrinsic edema-inducing activity when administered alone to skin sites. When PMSF-inhibited samples of CH were tested for edema-inducing activity in mouse skin, the level of edema was significantly lower (\(P < .001\)) than that formed with untreated CH (figure 2).

**Enhancement of worm burden with cercarial extracts.** Mice were infected with 50 \(\mu\text{L}\) of CH, cCTF, or SH before infection of the injection site by perfusion with 200 \(S.\ mansoni\) cercariae. Isotonic saline (IS) controls for the CH and SH groups and medium 199 controls for the cCTF group were included, as well as a normally infected group (control). On examination 42 days after infection, the perfused worm burden was significantly increased in the CH-injected mice compared with those of the control and IS-injected groups (\(P < .05\); figure 3). The worm burden in the cCTF-injected group was also significantly greater than in control infected animals (\(P < .05\)) but not the medium 199–injected group (figure 3). Injection of SH id failed to enhance worm burden.

Table 1 shows that if CH was injected id 24 h after cercarial application at the same site, there was still a significant enhancing effect on parasite infectivity (\(P < .05\)). Injection 48 h after or 24 h before percutaneous infection had no such effect. In

![Figure 1. Levels of edema in mouse skin following intradermal injections of cercarial homogenate (CH), concentrated cercarial transformation fluid (cCTF), schistosomula homogenate (SH), or saline. Larval extracts caused significant dose-dependent edema vs. saline treatment (* \(P < .05\); ** \(P < .001\)). Each group was 6 mice.](image-url)
Table 1. Effect of intradermal injection of saline or cercarial homogenate (CH) before or after infection with *S. mansoni* cercariae over the same site.

<table>
<thead>
<tr>
<th>Intradermal injection</th>
<th>Worm recovery, mean ± SE</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>45.2 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>At -24 h Saline</td>
<td>48.0 ± 4.1</td>
<td>NS</td>
</tr>
<tr>
<td>CH</td>
<td>49.3 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>At 0 h Saline</td>
<td>46.5 ± 2.5</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>CH</td>
<td>64.2 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>At +24 h Saline</td>
<td>43.3 ± 1.7</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>CH</td>
<td>65.8 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>At +48 h Saline</td>
<td>55.2 ± 3.3</td>
<td>NS</td>
</tr>
<tr>
<td>CH</td>
<td>52.5 ± 4.8</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. CBA/Ca mice were given CH 24 h before (~24), minutes before (0), 24 h after (+24), or 48 h after (+48) percutaneous application of cercariae as described in Materials and Methods; 6 mice were in each group. NS = not significant.
* Student’s *t* test of difference in worm burden between saline- and CH-injected groups.
† Saline-injected groups were not significantly different from noninjected control group.

Experiments involving administration of cercariae at sites distant from the site of id injection, there was no apparent enhancement of infectivity: control worm recovery, 47.3 ± 6.4; CH injected in flanks, 43.2 ± 8.2; CH injected in the abdomen, 62.3 ± 7.3 (*P* < .05).

Effect of protease inhibition on enhanced infectivity by cercarial extracts. Using a guinea pig skin model, we showed a correlation between the degree of inflammation induced by id injection of cercarial extracts and the amount of proteolytic activity in the injected extract. Furthermore, edema formation and eosinophil and neutrophil accumulation were significantly inhibited by pretreatment of the injected larval extracts with serine protease inhibitors, including PMSF and soybean trypsin inhibitor [10]. PMSF-inhibited samples of CH were tested in the id injection/percutaneous infection protocol described above. Figure 4A shows that id injection of PMSF-treated CH did not enhance infectivity of cercariae placed over the injection site, while mice injected id with untreated CH again had significantly higher worm counts than did control groups (*P* < .05). Similarly, preboiling of CH to inactivate enzymes before id injections completely removed the capacity of CH to enhance infectivity (figure 4B).

Effects of preformed inflammatory mediators and purified elastase on edema formation and schistosome infectivity. Inflammatory mediators known to induce edematous reactions were tested for their effects on schistosome infectivity in mouse skin. Bk and ZAP induced small but significant (*P* < .05) local edema compared with saline (figure 5A). In the presence of the vasodilator PGE₂ (which by itself did not cause leakage of plasma proteins), both Bk- and ZAP-induced edema responses were significantly enhanced (*P* < .01; figure 5B) to levels comparable with those caused by CH and cCTF (cf. figure 1). Injections of Bk or ZAP, alone or in combination with PG, just prior to percutaneous infection over the same skin site did not result in worm burdens statistically different from those in control groups (figure 5B), while the enhancing effect of id injection of CH was maintained (*P* < .05).
S. mansoni Proteases Enhance Infection

Figure 4. Effect of inhibition of protease activity in cercarial homogenate (CH) by pretreatment with PMSF or heat inactivation on infectivity of S. mansoni. Before percutaneous infection, mice were injected intradermally with (A) CH, PMSF-treated CH, isotonic saline (IS), or IS plus phenylmethylsulfonyl fluoride (PMSF) or (B) IS, CH, or heat-inactivated (boiled) CH. Inhibition was significantly increased (* P < .05) in CH-treated vs. other groups. Each group was 6 mice.

Commercial porcine pancreatic elastase was used in the id injection model to evaluate protease-induced edema in mouse skin. Mice were injected with CH or an equivalent quantity of pancreatic elastase (azocoll assays were done to ensure that comparable levels of protease activity were administered). Injection of CH or elastase evoked comparably significant levels of edema in mouse skin (P < .001), while pond and snail water caused levels of edema similar to that of the saline control (figure 6A). When CH, pancreatic elastase, and pond and snail water were tested for enhancement of infectivity, only CH and

Figure 5. Effect of intradermal (id) injections of inflammatory mediators on edema formation and infectivity of S. mansoni. A, Levels of edema in mouse skin following id injections of isotonic saline (IS), zymosan-activated plasma (ZAP), and bradykinin (Bk) with or without prostaglandin E2 (PG). Increase in edema was significant (* P < .05 and ** P < .001) vs. IS-treated group. B, Worm recoveries from mice exposed to S. mansoni. Before percutaneous infection, mice were injected id with IS, IS+ZAP, Bk+PG, Bk+PG, ZAP+PG, or cercarial homogenate (CH). Infectivity was significantly increased (* P < .05) in CH-treated vs. other groups. Each group was 6 mice.
elastase caused a significant increase in worm burden compared with that of isotonic saline–injected animals ($P < .01$), and pond and snail water did not affect infectivity (figure 6B).

**Discussion**

The mechanisms by which schistosome larvae gain access to the vascular system by migrating through the skin of their definitive host have not yet been precisely defined. It has been proposed that the proteolytic activities present in the acetabular gland secretions [14–18], combined with muscular activity of the parasite larvae, are important in this respect. Stirewalt [19] also suggested that a mucoid secretion was in part responsible for tissue damage that aided larval penetration. It has been shown that larvae migrating through skin cause an extravasation of high-molecular-weight proteins from blood vessels underlying the site of infection [9], and id injections of larval extracts cause similar extravascular leakage [2, 10]. In the present study, we have confirmed the edematous response induced by id injection of *S. mansoni* larval extracts in mouse skin and shown that CH, cCTF, and SH all induced edema formation, with CH and SH being more effective than cCTF.

Previous work from our laboratories has shown that id injections of cercarial extracts induced protease-dependent inflammatory responses in guinea pig skin [10]. In the present study, the edematous response induced in mouse skin by CH was reduced by 83% as a result of pretreatment of the extract with a concentration of PMSF that inhibited 79% of the azocollagenolytic activity in the extract. These results are consistent with the idea formulated previously [10] that the inflammatory activity in the larval extracts is mediated by one or more serine proteases. The main objective of the present study was to investigate further whether the inflammatory response so induced played any role in cercarial penetration in the mouse, a host that has been more widely used for host-parasite relationship studies than the guinea pig.

Intradermal injections of CH at the time and site of percutaneous application of a cercarial suspension consistently resulted in a significant increase in the number of adult worms retrieved by perfusion of the portal system 6 weeks later. The enhancing effect was dependent on the injection and infection being at the same skin site and was apparent even if the intradermal injection was given 24 h after the infection, though not if given 24 h before. It is known that larvae remain in the skin at the site of infection for 48–72 h before leaving via a blood or lymph vessel [20], and their migration could thus still have been affected by larval extracts injected 1 day after infection, while those given after 2 days would have been too late. The failure of injections given 1 day before infection to have any effect could be due to dissolution of the injected material in the intervening period.

The collagenolytic activity of CH could be totally inhibited by PMSF, indicating the presence of a serine protease, a finding consistent with that of other workers [7, 21]. In protease-substrate PAGE, we have found major collagenolytic activities in CH at 27–28 and 60 kDa (unpublished data), again a pattern similar to that found by others [6, 22–24]. PMSF pretreatment of the CH administered id resulted in inhibition of both the edematous reaction (figure 2) and of its infection-enhancing effect (figure 4B) in mice. Preboiling of CH (which completely removed all protease activity detected by azocoll assay) prior
to id injection obliterated the infection-enhancing ability of CH (figure 4B).

It has been generally accepted that with respect to schistosomes, the direct action of larval proteases on host skin tissue is essential for the infection process [2–4, 25]. The present results are consistent with this notion and reinforce the possibility of the use of protease inhibitors to suppress the infectivity of cercariae [25]. The discrepancy between the activities of CH and SH, as only the former enhanced infectivity, is paradoxical; however, both extracts induced comparable levels of edema (figure 1) and had similar levels of azocollagenolytic activity (data not shown). It may be that the protease activity profiles of schistosome larvae may be distinct at different stages of development [26–28].

While the present results indicate that enhancement of schistosome infectivity by id injections of CH was protease driven, infectivity of other organisms, such as the bacteria Proteus mirabilis [29] and Shigella flexneri [30], depends on induction of host inflammatory responses. A protease from Vibrio vulnificus has been directly implicated in enhancement of vascular permeability and edema formation [31]. From the results presented here, it cannot be determined whether enhancement of schistosome infection was due to the inflammatory response in the skin induced by the injected protease(s) or to direct proteolytic action on host tissue. Three separate results from this study could be considered suggestive of the latter possibility: Intradermal injections of Bk and ZAP, each in combination with PGE2 [32, 33], caused edematous reactions comparable in magnitude to those induced by injected CH, but these inflammatory mediators did not enhance infectivity; intradermal injection of commercial pancreatic elastase induced comparable levels of edema and enhancement of infectivity as did CH; and PMSF treatment or heat inactivation of protease activity in CH disabled the infectivity-enhancing effect of CH.

We are using biochemical techniques to isolate the major serine protease activities of S. mansoni larvae in order to test proteolytic fractions of greater purity in this system. A recombinant form of the cercarial elastase [26] (Price HP, personal communication) will also be evaluated for its effects on host immunity and schistosome infectivity. We are also investigating whether the compounds that inhibited the inflammatory responses induced by CH in guinea pig skin [10] (particularly Bk antagonist HOE 140, platelet-activating factor antagonist WEB 2086, and 5-lipoxygenase inhibitor PF 5901) can modulate the infection-enhancing effects of id injections of CH.

References


