Schistosoma mansoni: A chemoattractive factor released by males and its receptor in females

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Abstract. Chemoattraction between developing (23- to 28-day-old) and adult (10- to 12-week-old) *Schistosoma mansoni* males and females has been described previously. We report here on in vitro attraction of mature worms to released products of worms of the opposite sex. In the absence of a stimulus worms migrated randomly and did not show any preference in their orientation. Males were not significantly attracted to released products of females. Females exhibited greatest attraction to released products of males, and some attraction to the lipid fraction of released products of males. The aqueous fraction of male-released products slightly repelled females. Prior exposure to released products of males rendered females unresponsive to subsequent exposure, suggesting receptor saturation. These findings have important potential implicatons for the control of schistosomiasis by intercepting attraction and mating between males and females.

Key words. Schistosoma mansoni; chemoattraction; chemoattractant; chemoreception; chemoreceptors; lipids; pheromones; receptors; worms.

Schistosomiasis afflicts 200–300 million people throughout the world and causes up to one million deaths annually[1–3]. Biological and chemical methods for the control of the snail intermediate hosts have been unsuccessful. Despite enormous efforts, immunoprophylactic measures are not available[4]. Drugs for mass treatment are effective, although they remain unaffordable in many countries and humans get reinfected following chemotherapy[5]. Drugs are of limited use because of their toxicity and the ability of schistosomes to develop resistance to them[6–8]. Therefore, alternative approaches to the control of schistosomiasis are needed.

Schistosomes are dioecious trematodes (i.e., they have separate sexes). Humans are infected when cercariae penetrate intact skin and transform into juvenile worms called schistosomula[9]. Schistosomula reach the portal circulation by the hematogenous route. They begin mate-finding after approximately 23–28 days[10,11]. Pathogenesis in schistosomiasis is largely due to host immune reactions against eggs trapped in tissues[1,12]. Because females do not reach sexual maturity unless they are mated with males[13,14], efforts to interfere with mate-finding and thus egg-laying appear reasonable.

Chemoattraction studies using schistosome adults have been reported only recently[15–17]. The studies tested attraction between adult worms which had been separated immediately after they were recovered from the host. Extracts of females attracted males, as did lipid fractions of the extracts obtained using *n*-pentane and ether[18]. Analyses of the least polar fractions by thin-layer chromatography and nuclear magnetic resonance spectroscopy suggested that the major components were steroids, and the major steroid was cholesterol. However, authentic cholesterol did not attract[19]. These results are difficult to interpret because multiple responders were used, and interactions between responders may have influenced the outcome. Also, the number of observations was small.

We previously reported our preliminary observations on chemoattraction of schistosome adults to released products of worms of the opposite sex[20]. The studies described here extend the findings to aqueous and lipid fractions of released products in bioassays which have the advantage of measuring responses of indiviudal worms, and also describe the inhibition of chemoattraction by receptor saturation using worm released products.

Materials and methods

The parasite. A Puerto Rican strain of *Schistosoma mansoni* (PR-1) is routinely maintained in female CF-1 mice and an albino M-line of *Biomphalaria glabrata* snails. Mice were infected percutaneously by the application of cercariae to the shaved abdomen or by a tail-immersion method. They were killed by i.p. injection with 0.3 ml of a 1:1 mixture of 50 mg/ml sodium pentobarbital and 1000 units/ml sodium heparin. Adult worms were recovered from laboratory-infected mice by perfusion[21] in Earle's balanced salt solution (EBSS) containing 0.1% glucose and 0.5% lactalbumin hydrolysate (pH 7.0). Male and female adult worms were separated by gentle prodding and maintained in EBSS (4 °C) for approximately 10 min, then placed in bioassay channels.

Mice were handled in accordance with the 'Guide for the Care and Use of Laboratory Animals', National Research Council, National Institutes of Health, USA, Publication No. 86-23.

Collection of worm-released products. Worm-released products or their aqueous or lipid fractions were used. Released products were prepared by incubating individual males and females for 1.0 h in 100 µl of EBSS at 37 °C. Aqueous and lipid fractions were prepared by extracting

released products with *n*-hexane (v/v × 2). They were then pooled and dried under a stream of nitrogen and reconstituted in a known volume of EBSS by sonication. Untreated released products or reconstituted fractions were used to prepare agar cylinders.

The bioassay system. The bioassay system previously described[15] was used with the modifications mentioned below. It consisted of plexiglass chambers with 15 linear channels, each marked into five 'zones'. Each channel was 3.5 cm long, 1.0 cm wide, and 1.5 cm high, filled to a depth of 0.85 cm with 1.0% agar in phosphate buffered saline (PBS, pH 7.0). The agar was overlaid with 1.5 ml EBSS (pH 7.0). Experiments were done in a humidified incubator at 37 °C.

Because the original bioassay was designed to study chemoattraction between adult worms, it was modified to study worm attraction to released products of other worms. Agar cylinders were prepared by adding 75 µl of 2.5% agar in PBS (pH 7.0) at 40 °C to round-bottom 250-µl microtiter wells, then 100 µl of worm-released products or aqueous or lipid fractions, and finally another 75 µl of agar. After the agar had solidified, experimental agar cylinders and control cylinders containing 100 µl of EBSS were placed at opposite ends of alternating channels. A single worm of the sex opposite to the one supplying released products was placed equidistant between the experimental and control agar cylinders and allowed to migrate. To study the migration of worms in the absence of a stimulus, agar cylinders containing EBSS were placed at both ends of the bioassay channels. Worm migration was observed and recorded at 0.5-h intervals for 3.0 h[20]. Worms in the +2 and +1 zones were scored attracted, and those in the −1 and −2 zones were considered repelled. Worms in the 0 zone were not considered attracted or repelled.

Receptor saturation assay. Females were incubated individually for 1.0 h at 37 °C in 100 µl of male-released products, then placed in the bioassay channels, and their migration toward or away from a source of chemoattractant was observed at 0.5-h intervals for 3.0 h.

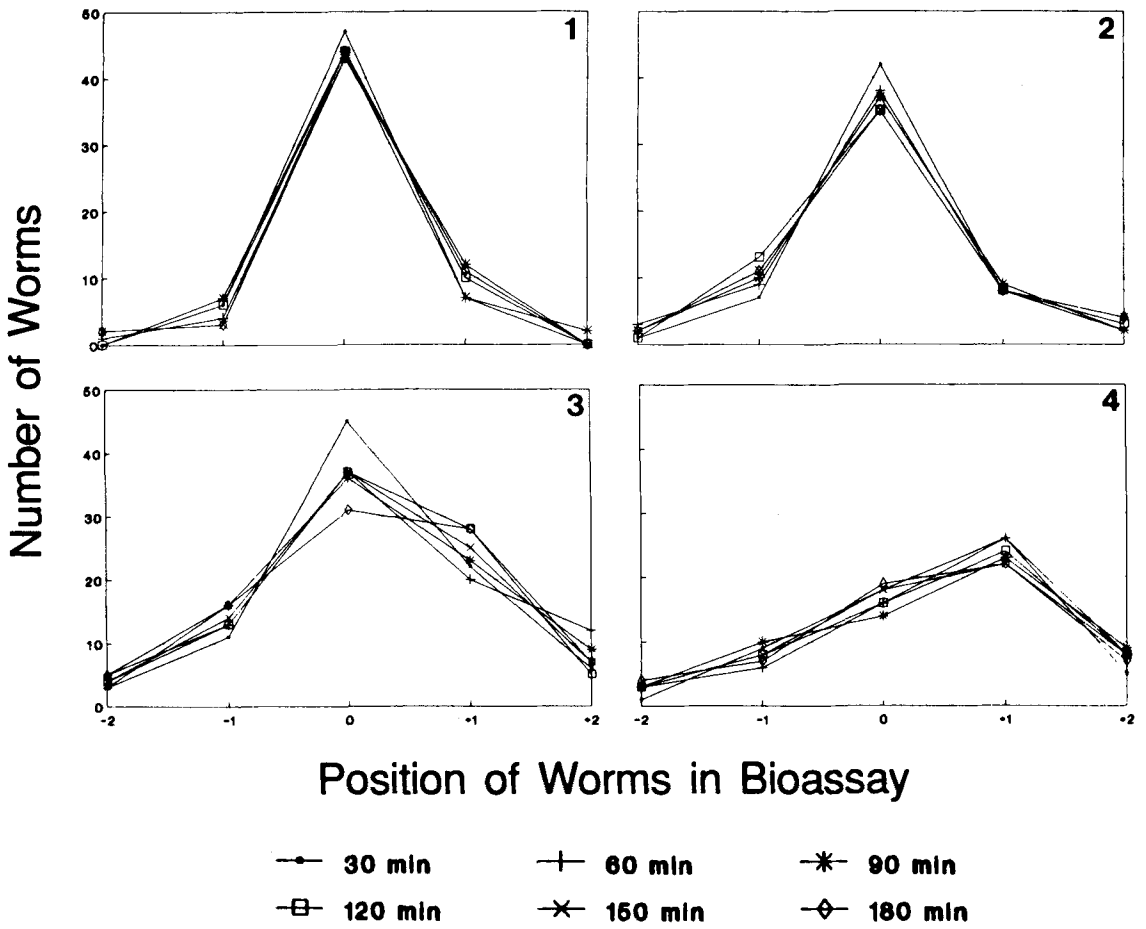

Figure 1. Distribution of males (n = 60) in the bioassay channels in the absence of a stimulus (agar cylinders at both ends were without worm products).

Figure 2. Distribution of females (n = 60) in the bioassay channels in the absence of a stimulus (agar cylinders at both ends were without worm products).

Figure 3. Distribution of males (n = 87) in the bioassay channels with agar cylinders containing unfractionated released products of females at the +2 end.

Figure 4. Distribution of females (n = 59) in the bioassay channels with agar cylinders containing unfractionated released products of males at the +2 end.

Data handling. Worm distributions were plotted (Harvard Presentation Graphics, Corporate Software, Canton, MA, USA) and compared using two-tailed Kruskal-Wallis ANOVA (True Epistat™, Epistat Services, Richardson, TX, USA).

Results

A summary of results of statistical analyses of data is presented in the table.

Random migration of worms. Random migration of males or females was studied in the absence of a stimulus by placing them in the '0' zone and allowing them to migrate. The majority of worms were found in the '0' zone at each observation time, and those which migrated into the positive and negative zones were approximately equal in number (figs 1 and 2). The distribution of males (n = 60) and females (n = 60) in the absence of a stimulus was similar ($p > 0.05$).

Chemoattraction to unfractionated released products. Males (n = 87) were not significantly attracted to released products of females when compared to their random migration ($p > 0.05$; fig. 3), even though after 1.0 h the total number of males in +1 and +2 zones was greater than those in −1 and −2 zones.

Females (n = 59) responded strongly to released products of males, and their distribution differed significantly from random migration throughout the observation period ($p < 0.05$; fig. 4).

Chemoattraction of females to lipid and aqueous fractions of released products of males. The aqueous fraction of male-released products did not attract females (n = 52), and actually resulted in a slight repulsion (fig. 5). The distribution of females was similar to that in the absence of a stimulus ($p > 0.05$) and significantly different from their response to unfractionated released products of males ($p < 0.05$).

Females (n = 60) were only slightly attracted to the lipid fraction of released products of males (fig. 6). This distri-

Probabilities (p) of differences between migration patterns of worms exposed to selected stimuli (Kruskal-Wallis ANOVA).

Time	A vs. B	A vs. C	B vs. D	B vs. E	B vs. F
30	0.826	0.106	0.005	0.301	0.195
60	0.178	0.358	0.000	0.018	0.532
90	0.597	0.233	0.006	0.074	0.741
120	0.315	0.182	0.002	0.062	0.639
150	0.322	0.401	0.012	0.192	0.381
180	0.387	0.316	0.020	0.371	0.608
Between groups	0.892	0.647	0.00000007	0.145	0.982

Time	D vs. E	D vs. F	B. vs. G	D vs. G
30	0.071	0.001	0.969	0.011
60	0.062	0.0008	0.818	0.001
90	0.171	0.008	0.868	0.014
120	0.105	0.002	0.985	0.024
150	0.135	0.004	0.372	0.007
180	0.151	0.016	0.742	0.042
Between groups	0.114	0.00000001	0.998	0.0000568

A, random migration of males (control);
B, random migration of females (control);
C, response of males to released products of females;
D, response of females to released products of males;
E, response of females to lipid fraction of released products of males;
F, response of females to aqueous fraction of released products of males;
G, receptor blockage in females using released products of males.

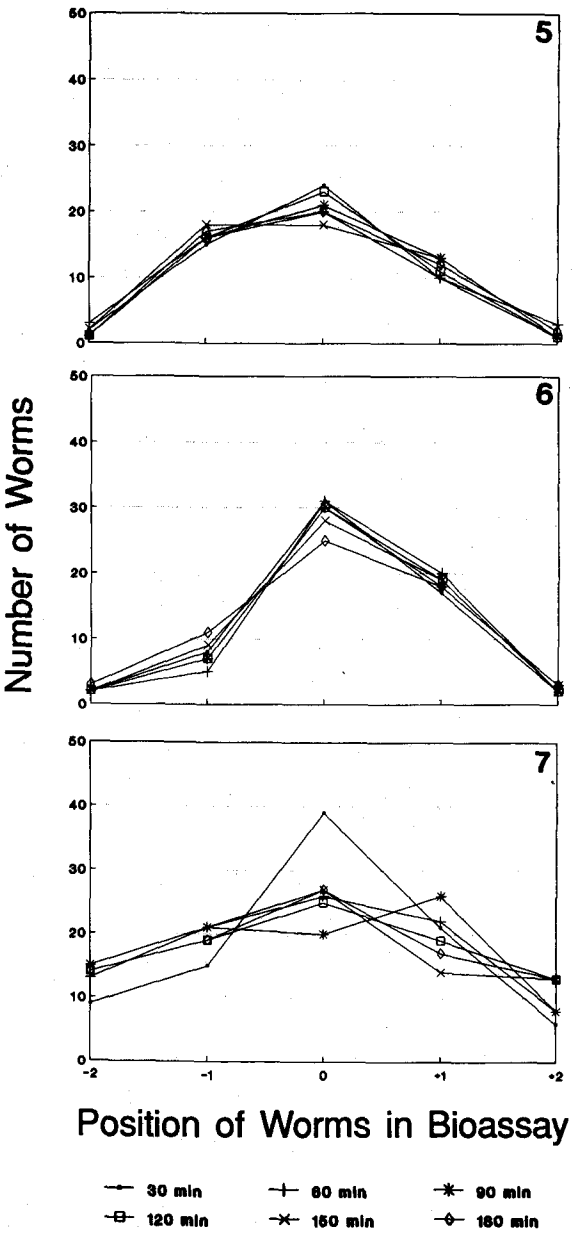

Figure 5. Distribution of females (n = 52) in the bioassay channels with agar cylinders containing aqueous fraction of released products of males at the +2 end.

Figure 6. Distribution of females (n = 60) in the bioassay channels with agar cylinders containing lipid fraction of released products of males at the +2 end.

Figure 7. Distribution of females (n = 90) previously incubated in released products of males for 1.0 h at 37 °C in the bioassay channels with agar cylinders containing unfractionated released products of males at the +2 end.

bution was similar to that of females in the absence of a stimulus or in response to released products of males ($p > 0.05$). However, at 1.0 h, the distribution of females differed from that in the absence of a stimulus ($p < 0.05$).

Receptor saturation assay. When females ($n = 90$) were preincubated in male-released products for 1.0 h, then tested in the bioassay for attraction to those products, their distribution was similar to that observed for random migration of females in the absence of a stimulus ($p > 0.05$; fig. 7). Although after 0.5 h females were relatively evenly distributed in bioassay channels, their distribution differed significantly from that of untreated females in response to male-released products ($p < 0.05$).

Discussion

Pheromones are substances emitted by organisms which can elicit specific behavioral or developmental responses in other individuals of the same or closely related species[22,23]. Although a specific pheromone(s) has not yet been identified for schistosomes, attraction between adult males and females has been demonstrated[15–17], and preliminary results on attraction to worm-released products have been reported[20] and reviewed[24].

This report extends our observations on attraction of single schistosome adults of one sex to released products of worms of the opposite sex. The data presented here demonstrate that *S. mansoni* males elaborate a chemical attractant for females, that the chemoattractant is lipophilic, and that chemoreception in females is receptor-mediated.

The results presented here contrast with previous reports of attraction of males to the lipid fraction of female-released products[18,19]. However, differences may be explained by the fact that the latter studies only used small numbers of worms, reported significant responses only after 24.0 h (compared with our findings within 1.0 h), and employed multiple responders while we studied responses of single worms.

Disruption of chemical communication in helminths by receptor saturation with the chemoattractant has been reported. After they were maintained in an environment permeated with pheromone produced by females, the ability of *Nippostrongylus brasiliensis* males to orient to a gradient of the pheromone was greatly reduced[25].

A comparison of worm-to-worm attraction and attraction of female worms to male-released products provides some information about the diffusion of released products in an aqueous medium. Maximal attraction to worms restrained in dialysis-tube chimneys[20] and worm-released products in agar cylinders (current study) was usually observed within 0.5 h. However, maximal attraction of females to lipid fraction of released products of males occurred at 1.0 h, which suggests that released products or the biologically active component(s) elaborated by worms are diffused earlier in an aqueous medium than the lipid component(s) of released products, and that the gradient disappears after 1.0 h. The ability of schistosome-elaborated materials to form in vitro gradients requires further investigation.

Lipophilic substances are only slightly soluble in water[26] and the mechanism(s) of their transfer in helminths is not known. The fact that labelled cholesterol and its metabolites can be transferred between adult males and females in vitro[27,28] argues for the diffusion of lipids in aqueous or semiaqueous environments[24].

When released products of males were treated with chloroform-methanol (2:1, v/v), a small amount of precipitate was observed at the interface of the lipid and aqueous fractions; the lipid fraction caused slight repulsion of females. However, treatment of male-released products with *n*-hexane did not produce precipitate and the resultant lipid fraction was attractive at 1.0 h ($p = 0.018$). The difference in worm responses to unfractionated released products and the lipid fraction is probably due to some loss of the pheromone during fractionation with chloroform-methanol.

Chemical characterization of the schistosome chemoattractant(s) may lead to the development of methods for interfering with chemical communication by blocking with chemical analogues, or vaccines against the chemoattractant or its receptor in females. Since it has been demonstrated that worms of one species attract those of other species[29], such chemical analogues or vaccines may be useful against all species of the genus *Schistosoma* which infect humans.

Tegumental membrane turnover has been the subject of several studies which used various tegumental antigens or components as markers for membrane turnover. Tegumental half-life differs between studies[30]. Since the receptors in females that mediate chemoreception can be blocked or saturated with the chemoattractant and a lectin (our unpublished data) previously known to bind to schistosome tegument, it is now feasible to study tegumental turnover using a marker with biologic function.

Contrary to previous reports of tegumental damage in schistosomes during in vitro maintenance[31,32], we have demonstrated that schistosomes do not sustain damage with careful handling and proper fixation[33]. It was also suggested that LAH, a constituent of EBSS, may damage schistosomes[31,32], but other studies have shown that LAH actually stimulates egg maturation and production in *S. mansoni* females maintained in vitro[34]. Lactoperoxidase-catalyzed radioiodination of surface proteins of *S. mansoni* adults in PBS yielded several labelled proteins. However, when worms were labelled under identical conditions in EBSS, no protein was labelled. It appears that when worms were incubated in PBS, internal proteins leaked into the medium where they became radiolabelled and were subsequently adsorbed by the tegument, whereas in EBSS, neither leakage nor labelling took place[30]. This finding offers further evidence that EBSS supports vital physiological activities of schistosomes in vitro.

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Lake Tanganyika as an evolutionary reservoir of old lineages of East African cichlid fishes: Inferences from allozyme data

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Abstract. Genetic differences between 20 species of cichlid fish, representing all the 12 tribes proposed for the cichlid fish fauna of Lake Tanganyika, were studied by allozyme electrophoresis. Most species were genetically very differentiated from each other. Phylogenetic analysis based on the allozyme data indicated that at least seven old, ancestral lineages have contributed to the present cichlid fauna of the lake. Lake Tanganyika, the oldest of the rift-valley lakes, can be recognized as an evolutionary reservoir of major lineages of cichlids in East Africa.
Key words. Cichlid fish; Lake Tanganyika; allozyme; genetic difference; phylogenetic relationship.

The presence of vast numbers of endemic species belonging to a single fish family, the Cichlidae, within each African rift-valley lake presents a unique challenge to evolutionary biologists[1–3]. Though considerable attention has been directed to the phenomenon, the general phylogenetic framework of these fishes has yet to be determined, primarily because of the difficulty of identifying morphological features for phylogenetic analysis.

Most cichlid species in Lakes Victoria and Malawi have been regarded as belonging to a single genus, *Haplochromis*, or being closely related to this genus[2]. Recently, Meyer et al.[4] examined mitochondrial DNA differences among representative cichlids in Lake Victoria and some in Lakes Malawi and Tanganyika. Lake Victoria cichlids were shown to be genetically quite similar to each other and more akin to those from Lake Malawi than