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Schistosoma mansoni: Silver Ion (Ag+) Stimulates and Reversibly Inhibits Lipid-Induced Cercarial Penetration

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KING, C. I., AND HIGASHI, G. I. 1992. *Schistosoma mansoni:* Silver ion (Ag+) stimulates and reversibly inhibits lipid-induced cercarial penetration. *Experimental Parasitology* 75, 31–39. Certain long-chain polyunsaturated fatty acids (FA) found on mammalian skin trigger cercariae to penetrate and transform into schistosomules; however, the mechanism by which FAs stimulate cercariae is unknown. In order to determine whether argentophilic papillae concentrated at the apical region of the cercariae are the chemoreceptors that may mediate cercarial response to FAs, an assay assessed the proportion of cercariae that penetrated a 0.25% agar matrix in the presence (61%) and the absence (2.3%) of linolenic acid at 0.22 mM. Silver nitrate (Ag^+) which selectively binds to cercarial papillae (Short and Cartrett, *J. Parasitol.* 59, 1041, 1973) is nontoxic (at 0.09 m*M* used in this study) as demonstrated by the ability of Ag^+ treated cercariae to mature successfully into adult worms (8.8% maturation compared to 10.2% of untreated controls, $n = 5$) after subcutaneous injection. When Ag^+ was added to cercarial suspensions, penetration into linolenic-impregnated agar was significantly inhibited (80.8%). Washing cercariae free of Ag^+ reversed this inhibition. These data, as well as observations that both argentophilic papillae and cercarial response to FAs disappeared within 3 to 4 hr after mechanical conversion to schistosomules, implicate argentophilic papillae on cercariae as chemoreceptors for lipid stimulation.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Schistosoma mansoni;* Cercariae; Chemoreceptors; Skin penetration; Silver nitrate; FA, fatty acid.

INTRODUCTION

Certain human infections are remarkable in that the parasite larvae penetrate directly into the intact human skin. Notable is schistosomiasis, estimated to infect 200 million people worldwide, in which free-living cercariae remain suspended in the fresh water of ponds and streams until they come in contact with the appropriate host. The cercariae then penetrate the skin. The exact mechanisms by which this process occurs remains poorly understood. The stimuli that trigger cercarial penetration into the skin, however, have been identified as unsaturated free fatty acids (FAs) found on the skin surface (Austin and Stirewalt 1972; Stirewalt 1971; MacInnis 1969; Shiff *et al.* 1972). In particular, certain long chain polyunsaturated FAs are the most active stimulants of cercarial penetration (Haas and Schmitt 1982a; Salafsky *et al.* 1984a). Putative mechanisms for FA-induced cercarial responses are (i) solubilization of the cercarial tegument by FAs that subsequently allow ion fluxes across the cercarial surface (Stirewalt 1971), (ii) passive diffusion of lipids across cell membranes to interact directly with cellular activating enzymes (e.g., protein kinase C, Matsumura *et al.* 1991), or (iii) interaction with specific receptors on the cercarial surface. The latter hypothesis is most attractive and directly testable for the following reasons. First, the

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relative effectiveness of different FAs to stimulate cercaria are largely independent of their polarity and water solubility (Haas and Schmitt 1982b) which suggests that they do not act by altering the cercarial tegument, but that they may interact with specific receptor sites on the cercarial surface. Second, cercariae possess surface papillae with ciliated nerve endings (Wagner 1961; Nuttman 1971; Short and Cartrett 1973; Short and Gagne 1975) that closely resemble the well studied chemosensory papillae observed in *Caenorhabditis elegans* (Perkins *et al.* 1986). Finally, a particular feature of these structures is preferential staining with silver nitrate (referred to as "argentophilic papillae") (Wagner 1961; Richard 1968a,b; Short and Cartrett 1973). We reasoned that silver nitrate may bind to protein moieties on papillae and ciliated nerve endings and thus prevent FA-induced cercarial penetration if these structures did, in fact, possess chemoreceptors. The present study demonstrates that silver nitrate in low concentrations is nontoxic to cercariae and can both stimulate and reversibly inhibit cercarial penetration responses.

Methods

Parasites. A Puerto Rican strain of *Schistosoma mansoni* was used throughout this study. Parasites were maintained in a laboratory adapted strain of *Biomphalaria glabrata* and outbred albino mice. Cercariae recovered from infected snails were thoroughly washed and exposed for 90 min to fluorescent light to induce shedding in spring water at 28°C. Cercariae were used within 2 hr of emergence.

Agar penetration by cercariae. Pure free fatty acids (Sigma, St Louis, MO) were emulsified by sonication in spring water, diluted to the appropriate concentration, and adjusted to pH 7.2 prior to use. Various FA emulsions were mixed with purified agar (Difco, Detroit, MI) to a final concentration of 0.25%, boiled and poured into 30 × 15-mm culture dishes (MacInnis 1969). Fresh cercarial suspensions were preincubated with various concentrations of silver nitrate (Eastman–Kodak Co., Rochester, NY) for 3 min and then added to the agar surface at room temperature (RT) for 1 hr; the agar surface was subsequently rinsed and both penetrated and unpenetrated cercariae were counted.

Cercarial preacetabular gland release. Approximately 200 freshly shed cercariae in 0.2 ml spring water were pipetted onto a glass slide into which FA emulsions were added to cercariae at various concentrations and incubated for 3 min at RT. The addition of 1 to 2 drops of a 5 mg/ml solution of pentobarbital immediately paralyzed the cercariae. Fifty microliters of 5% formalin was then added to fix the larvae. Pentobarbital inhibited contraction of the cercariae when fixed to prevent nonspecific release of their preacetabular gland contents. Cercariae were washed with distilled water and stained for 2 min with a saturated solution of purpurin stain prior to counting with a light microscope at 100×. Preacetabular gland contents which contains Ca^{2+} and stains red with purpurin were easily recognized throughout the length of the ducts to the excretory pores on the anterior portion of stimulated cercariae. In unstimulated cercariae, the stained preacetabular gland contents were confined to the glands. A response was graded as either positive or negative. Occasionally patches of stained material occurred along the ducts, but this could not be distinguished from an artifact of the fixing process and therefore not graded as a partial response.

Mechanical transformation of cercariae. Cercariae were transformed into schistosomules by rapidly passing the cercariae through a 21-gauge needle 14 times in Earle's medium (Colley and Wikel 1974).

Staining of papillae with silver nitrate. Cercariae were stained with hot (80°C) 2% silver nitrate and exposed for 20 min under a bright light prior to microscopic examination (Short and Cartrett 1973). The proportion of Ag^{+} stained papillae was determined by counting the mean number of argentophilic papillae on the cercarial body prior to transformation (mean, 61; $n = 3$) and determining the fraction of remaining papillae on schistosomules ($n = 3$) at various intervals after transformation.

Statistics. The results of three or four replicate experiments were pooled when appropriate and sample means compared by the Student's t test. Cercarial responses represent the difference between treatment values and the appropriate paired control value. Associations were determined by simple linear regression.

Results

Relationship of FA structure on cercarial responses. Long polyunsaturated or monosaturated FAs were weak stimulates of cercarial preacetabular gland release or cercarial penetration compared to structurally similar compounds with additional double bonds (Table I). For example, linolenic acid (0.22 m*M*) had approximately 10- and 5-fold greater ability to stimulate preacetabular

TABLE I
Relationship of Free Fatty Acid Structure on Cercarial Responsiveness

Fatty acid[a]	Structure[b]	% Preacetabular gland release	% Penetration
		Means ± SD	Mean ± SD
Palmitoleic	16:1	12.3 ± 1.3	1.1 ± 1.1
Steric	18:0	5.4 ± 2.2	0
Oleic	18:1	3.3 ± 0.9	0
Linoleic	18:2	7.8 ± 2.9	11.4 ± 1.5
Linolenic	18:3	98.2 ± 7.9	53.3 ± 3.2
Arachidonic	20:4	46.1 ± 3.2	17.2 ± 5.5
Docosahexaenoic	22:6	49.0 ± 3.7	61.7 ± 4.3

[a] Concentration of 0.19–0.24 m*M*.
[b] Lengh of carbon chain:number of double bonds.

gland release and agar penetration, respectively, than that of linoleic acid. The stimulatory effect of linolenic acid on preacetabular gland release and agar penetration was dose dependent (Fig. 1) as were linoleic, arachidonic, and docosahexaenoic acids (data not shown). Linoleic acid stimulated similar levels of preacetabular gland release (86%) and agar penetration (46%) as linolenic acid, but at the much higher concentration of 1.6 m*M*. Palmitoleic, steric, and oleic acids failed to stimulate agar penetration at any concentration, although palmitoleic (but not steric and oleic acids) stimulated 97.2% preacetabular gland release at a concentration of 2.0 m*M*.

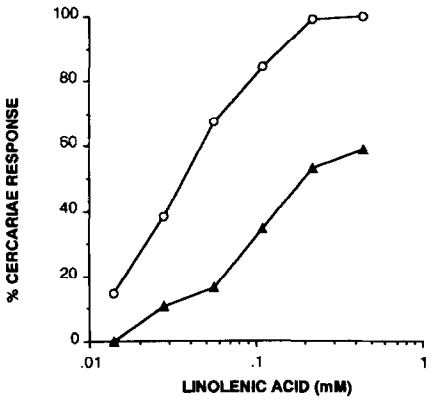

FIG. 1. Effect of different concentrations of linolenic acid on cercarial preacetabular gland release (open circles) and penetration (closed triangles). Each point represents the percentage of approximately 200 cercariae.

Fatty acids that stimulated significant preacetabular gland release also stimulated cercarial penetration; however, these two responses did not always correlate (Table I). In addition, no FA tested stimulated greater than about two-thirds of the cercariae to penetrate.

Because linolenic acid was the most potent stimulant for cercarial gland release tested and also a strong stimulant for cercarial penetration, it was used in all subsequent experiments at a concentration of 0.22 m*M*.

Effect of Ag^+ on cercarial preacetabular gland release and penetration. Ag^+ impregnated agar-stimulated cercarial penetration in a dose-dependent fashion with a peak response at 0.09 m*M* (Fig. 2). At concentrates up to 0.09 m*M* of Ag^+ less than 5% of cercariae released their preacetabular gland contents. Higher Ag^+ concentrations induced more preacetabular gland release (peak of 19.1%); however, these concentrations of Ag^+ were toxic to cercariae as determined by impaired cercarial motility, uptake of trypan blue, and failure to infect mice with patent infections after the Ag^+ solution was washed away. In contrast, Ag^+ concentrations of 0.09 m*M* and lower were nontoxic to cercariae as determined by (1) no impairment in activity; (2) persistent exclusion of trypan blue; (3) prolonged incubation with Ag^+ which did not alter its inhibitory effect; and (4) subcuta-

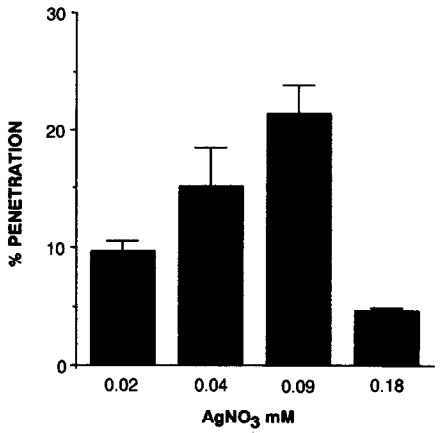

FIG. 2. Silver nitrate-impregnated agar stimulates cercarial penetration. The values represent the means ± SD of three separate experiments.

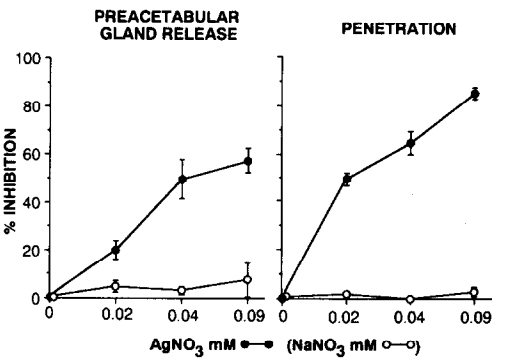

FIG. 3. Silver nitrate (solid circles) or $NaNO_3$ (open circles) inhibition of linolenic acid-induced preacetabular gland release (left panel) and cercarial penetration (right panel). The data are expressed as mean percentage of inhibition (±SD) of three separate experiments.

neous injection of mice with cercariae pretreated with Ag^+ and then washed, developed into adults with the same frequency as controls (8.2% vs 10.2% in controls; $n = 6$). The stimulatory effect of $AgNO_3$ appeared to reside in the Ag^+ ion since $NaNO_3$ failed to induce either preacetabular gland release or cercarial penetration.

The effect of Ag^+ on cercarial penetration behavior was similar, but not identical to that observed with lipids. Ag^+ failed to induce the same degree of increased cercarial activity or vigorous thrusting behavior when stimulated. Those cercariae that did penetrate also demonstrated similar, but less pronounced probing behavior than that observed in lipids. The failure of Ag^+ to stimulate strong thrusting and synchronized muscular contractions during penetration may account for the absence of preacetabular gland release.

Ag^+ inhibition of free fatty acid-induced cercarial responses. Low concentrations of Ag^+ in the cercarial suspension inhibited both cercarial preacetabular gland release and penetration into an agar matrix containing linolenic acid in a dose-dependent fashion (Fig. 3). The presence of Ag^+ (at 0.09 m*M*) also significantly inhibited (by 74.4%) cercarial penetration into agar containing docosahexaenoic acids. Of note, Ag^+ inhibited cercarial penetration to a greater extent than that of preacetabular gland release. The effect of Ag^+ suspensions on cercarial behavior was minimal, showing a slight increase in swimming activity; however, there were no significant preacetabular gland release or cercarial penetration responses. In control experiments, preincubation of cercariae with the same concentrations of $NaNO_3$ failed to inhibit either cercarial preacetabular gland release or penetration (Fig. 3).

Cercariae initially exposed to Ag^+ and subsequently washed twice in spring water to remove the Ag^+ were able to penetrate to a mean of 74% of unexposed cercariae (Fig. 4). Exposure of cercarial to Ag^+ for up to 1 hr had no effect on the degree of inhibition or the ability to reverse the inhibition when the Ag^+ was removed (data not shown).

Effect of cercarial transformation into schistosomules on penetration and the presence of argentophilic papillae. Mechanical transformation of cercariae into schistosomules resulted in a loss of argentophilic papillae on cercarial surfaces (Fig. 5). The values shown in Fig. 5 indicate the percentage of cercariae possessing any surface papillae and separately, the mean pro-

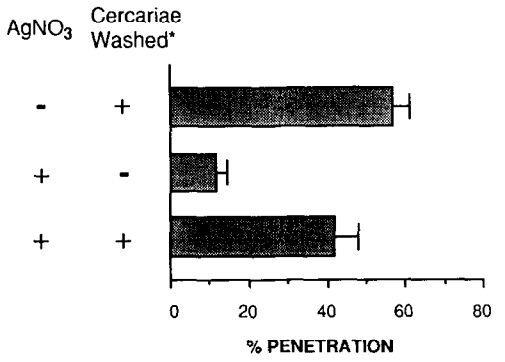

FIG. 4. Effect of exposing and subsequent removal of Ag^+ on cercarial ability to penetrate an agar matrix containing linolenic acid. The values represent the mean percentage of cercarial penetration (±SD) of seven separate experiments.

portion of argentophilic papillae remaining on an individual schistosomule. Argentophilic surface papillae decreased rapidly posttransformation and the diminution in the mean percentage of papillae remaining on schistosomules significantly correlated with a decreased capacity to penetrate into FA-impregnated agar (Fig. 5; $r^2 = 0.86$, $P < 0.01$).

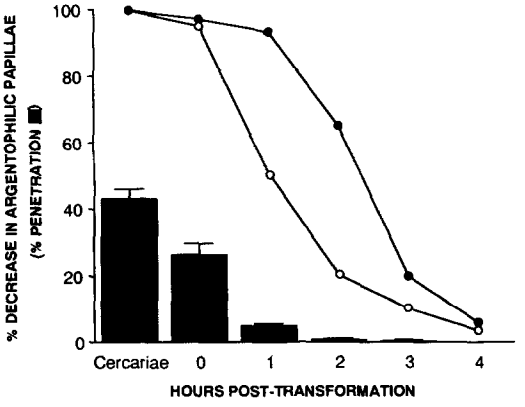

FIG. 5. Effect of cercarial transformation into schistosomules by tail removal on the penetration response into agar containing linolenic acid. The line with closed circles represents the percentage of cercariae with *any* argentophilic papillae. Open circles represent the mean percentage of argentophilic papillae remaining on individual cercariae. The bars represent the mean (±SD) percentage of cercarial penetration of two separate experiments.

DISCUSSION

Our major findings are that the heavy metal ion, Ag^+, can stimulate and also inhibit cercarial penetration into an agar matrix containing purified free fatty acids. Cercarial responses occur rapidly after Ag^+ exposure and may result from blocking and partial engagement of probable receptor sites for fatty acids on argentophilic papillae found on the larval surface. These conclusions are derived from the observations that Ag^+ appears to preferentially bind to sensory papillae, its effects are immediate, and the inhibition is rapidly reversed by washing the cercariae free of Ag^+.

The ability of cercariae to penetrate lipid-impregnated agar (MacInnis 1969; Haas and Schmitt 1982a) allows easy quantification and direct observation of cercarial behavior. Unpenetrated cercariae remained viable in contrast to most agar-penetrated cercariae that subsequently lost their tails, stopped moving, and eventually died, indicating that lipid-stimulated cercariae had also transformed into schistosomules. This failure of unpenetrated cercariae to die contrasts to the studies of Haas and Schmitt (1982a) and probably relates to the method of free fatty acid emulsification. The present study emulsified lipids with sonication, whereas Haas and Schmitt (1982a) used Tween 80 which probably enabled greater diffusion of lipids into the overlying spring water. As an additional criterion of cercarial stimulation, lipid-induced preacetabular gland release was determined by the presence of purpurin-staining material (Ca^{2+}) throughout the length of the excretory ducts, which was easily graded as either a positive or a negative response.

In none of the experiments did more than about two-thirds of the cercariae penetrate the agar matrix and often the proportion was significantly less. Initial contact of the agar surface by cercariae appeared to be by chance and unpenetrated cercariae may not

have made adequate contact to stimulate penetration. The failure of many cercariae to penetrate suggests that skin surface lipids, with their poor water solubility, may not attract cercariae to the host skin, but only provide a stimulus for penetration once cercariae have come into contact with the skin. Arginine has been suggested as an attachment stimulus for cercariae (Granzer and Haas 1986) whereas CO_2 and HCO_3 may be chemoattractants over longer distances (King 1984; Motzel and Haas 1985).

Other studies have also examined the relative capacity of different FA to stimulate cercariae. Haas and Schmitt (1982a), also using a lipid impregnated agar assay, demonstrated an order of response as follows: arachidonic > linolenic > palmitoleic > linoleic acids at similar concentrations (0.02 to 1.0 m*M*). Although these findings varied from the present study (from Table I: docosahexaenoic > linolenic > arachidonic > linoleic > palmitoleic), a difference probably attributable to the different methods of emulsification (see above), the overall pattern was similar in that the more highly unsaturated FA were the most potent for cercarial stimulation. In contrast, Salafsky *et al.* (1984b) found a different pattern of response with linoleic > arachidonic > palmitoleic acids based on an assay in which a suspension of cercariae accumulated to the center of a petri dish coated with a particular FFA of an unspecified concentration. The nature of the cercarial behavior measured in this assay was uncertain, whether chemotaxis took place or cercariae began penetration behavior after random contact with the fatty acid, made direct comparison with the present study difficult.

The mechanism of Ag^+ effect on cercariae responses is likely mediated through argentophilic or sensory papillae on the cercarial surface for the following reasons. First, Ag^+ appears to bind preferentially to surface papillae. Localization of Ag^+ to other parts of the nervous system or the cercariae have not been reported (Short and Cartrett 1973; R. B. Short, personal communication). Additional attempts to stain and section cercariae confirms the Ag^+ localization to surface papillae (personal observations). In preliminary studies, C^{14}-labeled linolenic acid appears to localize to papillae (specifically the pit receptors in the anterior portion of the cercariae) using autoradiography and light microscopy (King 1984). Second, cercarial responses to Ag^+ were immediate, suggesting its interaction with a superficial structure. Although acute Ag^+ inhibition has not been demonstrated for nerve cells, it can be seen with renal cortical (Kone *et al.* 1988) and heart muscle cells by induction of K^+, Na^+, and Ca^{2+} flux across cell membranes (Brunder *et al.* 1988; Prabhu and Salama 1990). It is unlikely that Ag^+ acts acutely on analogous cell types in cercariae (e.g., flame cells or muscle cells) because exposure of the cercarial to Ag^+ prior to agar penetration had no apparent inhibitory effect on their swimming or movement.

To exclude the possibility that Ag^+ is toxic to cercariae at the concentrations used in the present study it was demonstrated that (a) Ag^+ treated cercariae remained viable; (b) cercariae exposed to silver nitrate, washed, and subsequently inoculated into mice successfully matured into adult parasites; and (c) the inhibitory effect of silver nitrate on cercariae was reversible. Although total reversal of cercarial penetration was not achieved, it was impossible to wash thoroughly actively swimming cercariae which probably resulted in some silver nitrate remaining in the cercarial suspension.

Ultrastructural studies of sensory papillae in *S. mansoni* cercariae have identified at least three types of ciliated papillae: (i) elevated, unicilated papillae; (ii) ciliated pits; and (iii) elevated, nonciliated papillae with a small central knob (Nuttman 1971; Short and Cartrett 1973). The function of these papillae in trematodes remains poorly understood. However, they are structurally

similar to the chemosensory sensillia in the head, called the amphids, of the nematode *Caenorhabditis elegans* (Ward *et al.* 1975; Perkins *et al.* 1986). The amphids, also argentophilic, contain the ciliated dendrites of 12 sensory neurons plus 2 support cells called sheath and socket cells that resemble the ciliated pit papillae of schistosome cercariae (Ward *et al.* 1975). The ciliated pits of schistosome cercariae are cavities with surface pores that provide contact with the exterior and possess six cilia that extend from sheath-like cells which are adjacent to a basal body (Nuttman 1971). The principle anterior and lateral locations of these papillae on the cercarial body further suggest that they may be important chemosensory organs.

Although skin surface lipids stimulate penetration behavior and transformation into schistosomules, other environmental stimuli also induce cercarial transformation. Increasing saline concentration triggers surface transformation of cercariae (Samuelson and Stein 1989). Interestingly, this surface transformation was blocked when cercariae were exposed to the acetylcholinesterase inhibitor, eserine sulfate, during the brief interval when osmolarity was raised, implying that receipt of this signal was interrupted (Samuelson and Stein 1989). This suggests that osmolarity changes are mediated through the nervous system and possibly, sensory receptors. Phorbol esters (which directly activate protein kinase C) induce cercariae to probe and initiate penetration responses and enzyme release, although, unlike responses to fatty acids, the cercariae do not shed their tails (Matsumura *et al.* 1991). Protein kinase C (PKC) is a key intracellular mediator of receptor-transduced signals in many eukaryotic cells and may be an important second messenger to receptor-induced cercarial stimulation. Although Matsumura *et al.* (1991) speculate that lipids may act directly on protein kinase C, it is equally if not more plausible that FAs act through a receptor that both stimulates PKC and induces signals that result in penetration behavior as well as cercarial tail loss and transformation.

Recently Ca^{2+} uptake by cercariae has been identified as critical for proteolytic enzyme release from their preacetabular glands (Fusco *et al.* 1991) and in the presence of the Ca^{2+} ionophore, A23187, induced cercariae to produce strong muscular contractions and tail loss (Matsumura *et al.* 1991). More importantly, free fatty acids (e.g., linoleic acid) induce calcium influxes into cercariae (Fusco *et al.* 1991). The ability of Ag^{+} to induce Ca^{2+} release from muscle sarcoplasmic reticulum (Prabhu and Salama 1990) and influxes of extracellular Ca^{2+} (Van Driessche *et al.* 1990) suggests a physiologic link between Ag^{+} and lipid-induced cercarial stimulation. Although Ag^{+} induced little preacetabular gland release, Ca^{2+} uptake is necessary, but not always sufficient to induce cercarial responses (Fusco *et al.* 1991; Matsumura *et al.* 1991).

Lipids also induced cercariae to produce eicosanoids (Fusco *et al.* 1985) and inhibition of eicosanoid production significantly reduces cercarial penetration (Salafsky *et al.* 1984a). Other studies, however, have failed to demonstrate that eicosanoid inhibitors can block either proteolytic enzyme release (Matsumura *et al.* 1991) or cercarial transformation (Wiest *et al.* 1989), although penetration and cercarial transformation may be by distinct events (Fusco *et al.* 1986). Whether Ag^{+} can block lipid-induced eicosanoid production might help to examine the role of eicosanoid production on cercarial transformation and penetration responses.

Much in known about penetration behavior of schistosome cercariae and the substances that trigger these responses. However, the mechanisms of cercarial responses to lipid stimuli remains poorly understood. This study suggests that certain surface papillae are chemoreceptors

for lipid stimulation and proposes further experiments by which this association can be confirmed. A detailed understanding of how lipids stimulate cercariae could provide novel approaches to interrupting transmission of this parasite to humans.

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