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Schistosoma mansoni: Human skin ceramides are a chemical cue for host recognition of cercariae

Wilfried Haas *, Simone Haeberlein, Sabina Behring, Eveline Zoppelli

Institute for Biology, University Erlangen-Nuernberg, Staudtstr. 5, D-91058 Erlangen, Germany

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ABSTRACT

Schistosoma mansoni cercariae recognize the human host with a sequence of behavioral responses particularly to chemical host cues. After attaching to the skin surface, cercariae are stimulated by so far unknown skin components to hold enduring contact with the skin and to start creeping towards entry sites. We studied the chemical stimulus of human skin for the cercarial enduring contact response by fractionation of human and pig skin surface extracts and offering the fractions to the cercariae via membrane filters. Enduring contact was stimulated exclusively by ceramides, specific lipids of the uppermost skin layers. This chemical cue differs from the 6 chemical host signals used by *S. mansoni* cercariae in other behavioral steps of host invasion, and thus underlines the specialization of *S. mansoni* cercariae particularly in chemical host signals. All together, the enduring contact response of the cercariae is, like the other phases of host invasion, well adapted to the chemical properties of human skin.

1. Introduction

Schistosomes infect their vertebrate hosts with aquatic cercariae, which actively invade their host's epidermis. The survival of schistosomes fully depends on the cercarial invasion success and they have evolved complex behavioral mechanisms to find, recognize, and invade their specific hosts (reviewed by Haas, 1994, 2003; Haas and Haberl, 1997). Each of the schistosome species studied so far shows its individual strategy of host-finding and host invasion, and this behavioral diversity seems to reflect adaptations to different ecological conditions within the habitats in which the parasites identify and invade their specific hosts. A particularly complex host-finding behavior is displayed by cercariae of *Schistosoma mansoni*. They show a high sensitivity and specificity primarily to chemical host signals during their host-finding. Six different chemical host cues have been described so far, which stimulate the parasites during the sequence of their host-finding and host-invasion phases: When *S. mansoni* cercariae have entered their host's active space, they swim towards the skin surface by responding to host-emitted fatty acids, L-arginine, and L-arginine-containing peptides (Haeberlein and Haas, 2008) and then attach to the skin responding to warmth and L-arginine (Haas, 1976; Granzer and Haas, 1986). They remain on the skin surface responding to warmth (Haas et al., 1994), but also to a chemical host signal, which has not been analyzed so far. The cercariae then search for entry sites following temperature gradients and concentration gradients of L-arginine (Haas et al., 1994, 2002), penetrate into the skin stimulated by defined fatty acids (Austin et al., 1972; Shiff et al., 1972; Haas and Schmitt, 1982a,b), and secrete the proteolytic enzymes with the acetabular gland contents responding to defined fatty acids (probably at the skin surface) and to glucosylceramides and phospholipids (probably in deeper skin layers) (Haas et al., 1997). Also after transformation to schistosomula, the larvae orientate along gradients of chemical host cues: L-arginine-residues and D-glucose seem to guide them through skin tissues towards venules (Grabe and Haas, 2004).

In the present study we identified the chemical cue of human skin which stimulates *S. mansoni* cercariae to remain on the skin surface after they have attached to it, and we found a cue (ceramides) which was particularly advantageous for this enduring contact ("remaining") response.

2. Materials and methods

2.1. Parasites

The strains of the human parasite *S. mansoni* and its intermediate host snail *Biomphalaria glabrata* were originally isolated in

* Corresponding author. Fax: +49 9131 8528040.
E-mail address: whaas@biologie.uni-erlangen.de (W. Haas).

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Belo Horizonte, Brazil. The parasite was maintained in white mice infected by the paddling method.

2.2. Experimental conditions

Influence of chemical host signals on the enduring contact behavior of cercariae was studied with methods modified after Haas (1976) and Granzer and Haas (1986). Briefly, up to 100 cercariae in buffered tap water (pH 7.0, 5 mM phosphate buffer) were pipetted into plastic spectrophotometer cuvettes ($10 \times 10 \times 40$ mm) whose temperature was adjusted to 25 °C with special cell holders. Test substrates were presented to upward swimming cercariae 2 mm below the water surface via horizontally positioned membrane filters glued to the lower ends of vertically fixed glass tubes (5 mm diameter). The cercarial responses were observed using horizontal dissecting microscopes and the cuvettes were illuminated at 50 W/m^2 with two cold light sources (KL 1500, Schott, Mainz, Germany).

Upward swimming cercariae usually touched the test substrates with their tails, and many of them performed attachment responses as described by Haas (1976) and Granzer and Haas (1986). In typical attachment responses, cercariae shift from backward swimming (tail first) to forward swimming (body first), swim in an arc back to the encountered substrate and attach themselves with the oral sucker. When cercariae remained on the substrate, they usually crept on the surface and some showed penetration attempts. Only individuals, which had performed complete attachment responses, were considered as candidates for enduring contact behavior. Cercarial enduring contact responses were quantified by determining the percentage of cercariae which crept on a substrate for at least 5 s after attachment. In one replicate the cercarial reactions were observed for 5 min (Table 2A) or up to 10 min (others) after substrate presentation. Then a new batch of cercariae and a new substrate were used. In some experiments, the duration of cercarial enduring contact was measured up to 120 s, and additionally the total number of cercariae remaining on the membrane after 5 min incubation time was recorded.

Tests with various types of filters and substrates revealed two methods, which were particularly suitable for the experiments in this study: Membrane filter method 1: The test substrates were offered to the cercariae as liquids passing through membrane filters (Millipore HABG, pore size 0.45 μm; Millipore, Eschborn, Germany). Each substrate solution (pH 7.0) contained 0.1 mM L-arginine (as attachment stimulus) and 5 mM phosphate buffer in addition to the substrates. Lipids were emulsified in water by sonication after addition of an equal amount of ethanol. All substrates per series were treated similarly.

Membrane filter method 2: Lipids were spread on the surface of filters (Durapore GVWP 0.22 μm, Millipore, Eschborn, Germany) and the attachment stimulus (0.01 mM L-arginine, pH 7.0, 5 mM phosphate buffer) passed through the hydrophilic filter membranes. The lipids were applied to the filters by pipetting 10 μl of the particular lipid (fractions from 0.5 mg/ml human skin surface lipids) in chloroform/methanol (2:1, v/v) directly on the filters which were glued in the same way to the glass tubes as described above. To improve the optical contrast to the cercariae, the membranes were previously colored for 1 min in a chloroform/methanol solution (2:1, v/v) containing black permanent ink, washed in chloroform/methanol (2:1, v/v), and air-dried. The control substrate was pure chloroform/methanol (2:1, v/v). After the solvent from substrate application was evaporated, 200 μl of the L-arginine solution was pipetted into the glass tube, and the prepared tube was immersed into a cuvette containing the cercariae as described above.

As cercariae are very sensitive to temperature gradients, all beakers containing cercarial suspension, L-arginine or substrates were maintained in a 25 °C water bath. The sensitivity of different cercarial populations (also from the same host-snail samples) to attachment and enduring contact stimuli varies from day to day and it differs enormously between winter and summer season (although the hosts were maintained under constant temperature and lighting conditions) and furthermore it depends on the age of the parasites. Therefore, cercariae were used for experiments within 3 h after being shed from snails, and all quantitative comparisons were done using the same cercarial populations. All experiments used a blinded protocol; the test substrates were given a code unknown to the microscopist observers.

2.3. Preparation and analysis of skin substrates

2.3.1. Human skin surface extracts

Complete human skin surface extract was obtained by rubbing distilled water vigorously on the skin surface of arms, breast, and back of volunteers and removing the formed emulsion with a blade. The emulsion was separated into lipophilic and hydrophilic extracts with methods according to Folch et al. (1957) as modified by Haas et al. (1987). Crude skin surface lipids were obtained by washing the skin of volunteers with ethanol. The lipids were fractionated by either, preparative thin-layer chromatography or solid phase extraction. Completeness of lipid fractionation was checked using a dot blot of refractionated lipids.

2.3.2. Preparative thin-layer chromatography (TLC)

TLC was performed using pre-coated plates (silica gel 60, layer thickness 500 μm, Merck, Darmstadt, Germany) and a chloroform/methanol/acetic acid (190:9:1, v/v/v) solvent system (Wertz et al., 1985). Six lipid fractions were obtained: nonpolar lipids (including diacylglycerols, triacylglycerols, wax esters, sterol esters, and hydrocarbons), cholesterol, free fatty acids, glycolipids, phospholipids, and ceramides. Fractionated lipids were visualized with 0.2% 2,7-dichlorofluorescein under ultraviolet light, scraped off from the plates, resolved in *n*-hexane (nonpolar lipids) or chloroform/methanol (2:1, v/v) (others), and separated from the silica gel by centrifugation. Pureness of the fractions was checked by TLC using the polar solvent system chloroform/methanol/water (40:10:1, v/v/v) and the nonpolar solvent system described by Haas et al. (1987). In brief, precoated TLC-plates (silica gel 60, layer thickness 250 μm, Merck, Darmstadt, Germany) were developed successively in n-hexane (to 19 cm), toluene (to 19 cm), and twice in n-hexane/diethyl ether/acetic acid (70:30:1, v/v/v) (to 10 cm). Lipids were visualized using 75% sulphuric acid and heating at 160 °C.

2.3.3. Solid phase extraction

Chromabond® Silica/NH_2 columns (3 ml, 500 mg aminopropyl phase, Macherey-Nagel, Düren, Germany) were pre-conditioned with 10 ml n-hexane. Then 4 mg of human skin surface lipids, dissolved in chloroform, were loaded onto the column and eluted successively with different solvent systems (in brackets) to obtain the following 6 lipid fractions (Bodennec et al., 2000): nonpolar lipids (5 ml n-hexane/acetic acid ethyl ester, 85:15, v/v), ceramides (4 ml chloroform/methanol, 23:1, v/v), free fatty acids (2 ml diethyl ether/acetic acid, 98:5, v/v), glycolipids (6 ml acetone/methanol, 9:1.35, v/v), neutral phospholipids (3 ml chloroform/methanol, 2:1, v/v), and acid phospholipids (chloroform/methanol/3.6 M ammonium acetate aq., 30:60:8, v/v/v). Pureness of the lipid fractions was checked as described for preparative TLC fractions. All fractions were dried with N_2 under ice cooling and dissolved in chloroform/methanol (2:1, v/v) to a lipid concentration equivalent to 0.5 mg/ml human skin lipids. Before this, the