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Disruption of ERK signalling in *Biomphalaria glabrata* defence cells by *Schistosoma mansoni*: Implications for parasite survival in the snail host

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Summary
Biomphalaria glabrata is an intermediate snail host for the human blood fluke *Schistosoma mansoni*. To survive in *B. glabrata*, *S. mansoni* must suppress the snail's haemocyte-mediated defence response; the molecular mechanisms by which this is achieved remain largely unknown. We report here that *S. mansoni* excretory–secretory products (ESPs) attenuate phosphorylation of extracellular signal-regulated kinase (ERK) in haemocytes from a *B. glabrata* strain susceptible to *S. mansoni*. Whole *S. mansoni* sporocysts also impair ERK signalling in these cells. In striking contrast, ERK signalling in haemocytes from a *B. glabrata* strain refractory to schistosome infection is unaffected by ESPs or sporocysts. Effects of ESPs on ERK are similar in the presence or absence of snail plasma, thus ESPs seem to affect haemocytes directly. These findings reveal novel schistosome interference mechanisms; as ERK regulates various haemocyte defence reactions, we propose that disruption of ERK signalling in haemocytes facilitates *S. mansoni* survival within susceptible *B. glabrata*.

Introduction

Like other molluscs, the snail *Biomphalaria glabrata* has a potent internal defence system enabling protection against intruding pathogens. Macrophage-like immune cells called haemocytes play the major part in surveillance and removal of intruders, assisted by humoral components such as carbohydrate-recognition proteins (lectins) and proteinase inhibitors [1–3]. Haemocyte-mediated defence reactions that facilitate killing of intruding organisms include phagocytosis, encapsulation, and the production of reactive oxygen and nitrogen intermediates [1,4,5].

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Biomphalaria glabrata is the main intermediate host for *Schistosoma mansoni*, the digenean parasite that causes human intestinal schistosomiasis in South America and sub-Saharan Africa. Within the snail, asexual development of the schistosome larvae takes place, with miracidia transforming into mother and daughter sporocysts before emerging as free-swimming cercariae. To survive and replicate in *B. glabrata*, *S. mansoni* must subvert the snail's internal defence response. The interference hypothesis [6] proposes that schistosomes actively disrupt haemocyte function, thus preventing parasite elimination. Functional studies done in the 1990s support this notion; excretory–secretory products (ESPs) from schistosome larvae impair haemocyte motility [7], protein synthesis [8], phagocytosis [9], and superoxide production [10] by haemocytes.

Recently, studies have focused on the molecular mechanisms that regulate molluscan haemocyte defence responses when haemocytes are challenged with a variety of immunomodulatory compounds (reviewed in [11]). In snails, such research has focused largely on conserved cell-signalling pathways such as the protein kinase C (PKC) [12–14], phosphatidylinositol 3-kinase (PI3-K) [15–17], and the mitogen-activated protein kinase (MAPK) pathways, including p38 MAPK [16,17] and extracellular signal-regulated kinase (ERK) [16–19]. This research has revealed that PKC regulates processes such as nitric oxide (NO) and hydrogen peroxide (H_2O_2) production in haemocytes [14,16,19] and cell spreading in a *B. glabrata* embryonic (Bge) cell line [13]. In addition, H_2O_2 output and phagocytosis have been shown to be regulated by p38 MAPK and PI-3K, with the latter also playing a part in cell spreading [15–17]. The ERK signalling module also appears to regulate multiple defence reactions in snail haemocytes; ERK has been found to co-ordinate NO and H_2O_2 output [16,19], phagocytosis [18], and cell spreading and aggregation [17]. Crucially, pharmacological inhibition of ERK also substantially impairs the encapsulation of *S. mansoni* sporocysts by *B. glabrata* haemocytes [17].

Work carried out by us using the model snail *Lymnaea stagnalis* (host to avian schistosomes) demonstrated that fucose and galactose, sugars that predominate on the schistosome surface tegument, down-regulate the activities of ERK and PKC in haemocytes [20]. This finding inspired us to propose a timely extension to the original interference hypothesis, that is, to survive, differentiate and replicate in their snail hosts, schistosome larvae disrupt the activities of key cell-signalling pathways, thus limiting the snail defence response [11]. In the present work, we have employed the *B. glabrata*–*S. mansoni* host–parasite system to study the effects of schistosome ESPs and schistosome sporocysts on ERK activity in haemocytes. Importantly, snail strains that are either susceptible or refractory to *S. mansoni* infection have been used; thus the effects of *S. mansoni* components on signalling processes in host defence cells have been explored in the context of host phenotype. We report that ERK activity is down-regulated by ESPs and by fixed sporocysts in haemocytes derived from susceptible snails; in striking contrast, this phenomenon is not observed with haemocytes derived from the resistant strain. Thus we appear to have uncovered a novel schistosome-mediated immune interference mechanism that might facilitate the survival of *S. mansoni* within its intermediate host.

Materials and methods

Snails and haemolymph extraction

Resistant (NHM Accession number 3017, derived from BS90 [21]) and susceptible (NHM Accession number 1742) *B. glabrata* strains were used. Snails were maintained in an incubator at 26 °C on a 12 h:12 h light–dark cycle in trays containing water filtered through a Brimak/carbon filtration unit (Silverline Ltd, Winkleigh, UK); water was changed weekly and snails were fed fresh lettuce twice a week. For experiments, adult snails were washed with distilled water, dried, and haemolymph from several snails of each strain extracted by head–foot retraction. Haemolymph was then pooled on ice and mixed with 0.5 vol of sterile Chernin's balanced salt solution [22] containing glucose and trehalose (1 g/l each; pH 7.2; CBSS).

In vitro transformation of *S. mansoni* and collection of ESPs

Livers were obtained from *S. mansoni* (Belo Horizonte strain)-infected mice and miracidia were hatched from eggs in spring water and collected. They were then washed three times each in spring water and CBSS containing penicillin, and streptomycin (100 U/ml of each; $CBSS^+$), using a Steri-cup HV filter unit (Millipore, Watford, UK) with a 0.45 μm membrane. A control sample was then prepared by pelleting ice-cold miracidia at 10,000*g* and collecting the supernatant; the supernatant was then concentrated as described below for ESPs, and frozen at −20 °C until required. This control preparation was used to check whether residual mouse liver components, that might be present after isolation of *S. mansoni* eggs and hatching of miracidia, influence ERK signalling in haemocytes. For ESP generation, washed miracidia were enumerated before being cultured in $CBSS^+$ in 25 cm^2 vented sterile tissue culture flasks (Nunc, Rochester, USA) at 26 °C for 36–40 h. The cultures were observed with an inverted microscope to ensure that the miracidia had transformed into sporocysts and were alive (i.e. sporocyst movement and/or flame cell movement was visible), the flasks were then shaken to remove any loosely attached miracidial ciliated plates from the sporocyst's surface. After allowing sporocysts to settle on the base of the flask, the culture medium was collected and centrifuged briefly at 10,000*g* to pellet miracidial plates before being syringe filtered (22 μm; Nalgene, Hereford, UK). The resultant ESP (and control, above) preparation was concentrated approximately 10–20 times at 4 °C in Vivapore 10 ml concentrators (Vivascience, Sartorius, Epsom, UK) with a 7500 MW cut-off; aliquots were then stored at −20 °C until required. The protein concentration of the ESP preparation was determined using the NanoOrange fluorescence-based assay kit (Molecular Probes, Leiden, Netherlands) and a Fluorstar Optima microplate spectrofluorometer (BMG Labtech, Aylesbury, UK) with bovine serum albumin (BSA) as the protein standard. Transformed sporocysts were collected and fixed for 30 min in 2% (v/v) formaldehyde. Free aldehyde groups were then blocked by overnight incubation in 1% (v/v) glycine at 4 °C; such fixing of sporocysts does not interfere with their ability to elicit the production of