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Resistance of *Biomphalaria glabrata* 13-16-R1 snails to *Schistosoma mansoni* PR1 is a function of haemocyte abundance and constitutive levels of specific transcripts in haemocytes ☆

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ABSTRACT

Continuing transmission of human intestinal schistosomiasis depends on the parasite's access to susceptible snail intermediate hosts (often *Biomphalaria glabrata*). Transmission fails when parasite larvae enter resistant individuals in wild snail populations. The genetic basis for differences in snail susceptibility/resistance is being intensively investigated as a means to devise novel control strategies based on resistance genes. Reactive oxygen species produced by the snail's defence cells (haemocytes) are effectors of resistance. We hypothesised that genes relevant to production and consumption of reactive oxygen species would be expressed differentially in the haemocytes of snail hosts with different susceptibility/resistance phenotypes. By restricting the genetic diversity of snails, we sought to facilitate identification of resistance genes. By inbreeding, we procured from a 13-16-R1 snail population with both susceptible and resistant individuals 52 lines of *B. glabrata* (expected homozygosity ~87.5%), and determined the phenotype of each in regard to susceptibility/resistance to *Schistosoma mansoni*. The inbred lines were found to have line-specific differences in numbers of spreading haemocytes; these were enumerated in both juvenile and adult snails. Lines with high cell numbers were invariably resistant to *S. mansoni*, whereas lines with lower cell numbers could be resistant or susceptible. Transcript levels in haemocytes were quantified for 18 potentially defence-related genes. Among snails with low cell numbers, the different susceptibility/resistance phenotypes correlated with differences in transcript levels for two redox-relevant genes: an inferred phagocyte oxidase component and a peroxiredoxin. Allograft inflammatory factor (potentially a regulator of leucocyte activation) was expressed at higher levels in resistant snails regardless of spread cell number. Having abundant spreading haemocytes is inferred to enable a snail to kill parasite sporocysts. In contrast, snails with fewer spreading haemocytes seem to achieve resistance only if specific genes are expressed constitutively at levels that are high for the species.

1. Introduction

Schistosomiasis is a group of chronic parasitic diseases afflicting at least 240 million people in over 70 countries (United States of America Centers for Disease Control and Prevention, http://www.cdc.gov/parasiteSchistosomiasis/index.html). Its causative agents, trematodes of the genus *Schistosoma*, cycle between humans and fresh water snails of which *Biomphalaria* spp. are important in Africa and South America. Infected humans discharge eggs which hatch upon contact with fresh water, releasing miracidia. These penetrate the snail headfoot/mantle and transform into primary sporocysts near their point of entry. Within susceptible snails, two generations of asexual reproduction result in the production of thousands of cercariae that, when shed from a snail, can infect humans. Susceptible intermediate snail hosts provide protection and nourishment for larval schistosomes as they multiply. In resistant *Biomphalaria glabrata* snails, which do occur in nature (Newton, 1953; Michelson and DuBois, 1978), the *Schistosoma mansoni* parasite fails to develop, presumably due to recognition and aggressive activities of the immune system.

Since humans must be exposed to snail-derived larvae in order to be infected with blood flukes, it is crucial to understand the mechanisms which permit or prevent the parasite's establishment in the snail. Considerable advances have been made toward this goal (Bayne, 2009; Loker, 2010; Moné et al., 2010; Martins-Souza

☆ *Note:* Nucleotide sequence data first reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers JZ482247 to JZ482252.

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et al., 2011; Hanington et al., 2012; Mitta et al., 2012; Negrão-Corrêa et al., 2012; Blouin et al., 2013; Ittiprasert et al., 2013), yet for both the recognition and the effector phases of the snails' defence responses, much remains to be learned. Among known facts are that snail size can influence infectivity rates: some strains of *B. glabrata* are susceptible as juveniles but resistant as adults (Richards et al., 1992), and larger snails exposed to *S. mansoni* have lower infection levels than smaller snails of the same age (Niemann and Lewis, 1990). Circulating snail haemocytes play a key role in immune surveillance (Oliveira et al., 2010) and will migrate from the haemolymph into the tissues after parasitic infection (Noda and Loker, 1989; Martins-Souza et al., 2009; Barçante et al., 2012). This change is most intense in resistant snails in which larger haemocytes nearly disappear from the haemolymph, while small cells gradually increase (Martins-Souza et al., 2009). Haemocytes are involved in parasite recognition (Negrão-Corrêa et al., 2012), a capability that involves carbohydrate-binding receptors on spreading haemocytes (Fryer et al., 1989; van der Knaap and Loker, 1990; Renwrantz and Richards, 1992; Johnston and Yoshino, 2001; Castillo et al., 2007; Martins-Souza et al., 2011; Mitta et al., 2012). These cells are phagocytic granulocytes and, in resistant snails, they encapsulate schistosomes and the parasites are killed.

In the process of encapsulation, carbohydrate ligand binding by haemocyte receptors initiates production of toxic reactive oxygen species (ROS) (Hahn et al., 2000; Humphries and Yoshino, 2008) and nitric oxide (NO) (Hahn et al., 2001). Additional evidence for the role of ROS in parasite killing is the association of the B allele of the cytoplasmic *Cu/Zn superoxide dismutase* (*sod1*) gene in *B. glabrata* with resistance to *S. mansoni*, and the fact that snails with the B allele have significantly higher *sod1* expression (Bender et al., 2007). Schistosomes produce quantities of proteins that can scavenge ROS, possibly a strategy to avoid oxidative damage (Mourão et al., 2009). Considered together, these facts strongly implicate oxidative stress and nitration in haemocyte-effected snail defences against schistosomes.

As a path toward examining the genes involved in determination of snail resistance (R) or susceptibility (S), we used the hermaphroditic nature of *B. glabrata* and its ability to self-fertilise to derive more than 50 inbred snail lines from our 13-16-R1 population. The lines, exhibiting approximately 87.5% homozygosity, were phenotyped for resistance *to S. mansoni* (PR1 strain). The most highly resistant (R) and most highly susceptible (S) lines were further examined for haemocyte numbers and for expression of a panel of genes known to be involved in oxidative stress or otherwise implicated in the R/S phenotype. We hypothesised that higher numbers of haemocytes, or haemocytes which constitutively express relevant defence genes at higher levels would successfully thwart trematode infection. Innate resistance of *Aedes aegypti* mosquitos to Dengue virus is similarly thought to rely on 'basal-level immune activation' of immune-related genes (Sim et al., 2013).

Because the R/S phenotype is influenced by snail size (Richards and Merritt, 1972; Richards et al., 1992), we counted spread haemocytes in small juvenile snails and in larger adults, and determined R/S phenotypes in those two size classes. When snail size, spread haemocyte number, and constitutive haemocyte mRNA levels of selected genes were considered alongside the R/S phenotypes, complex relationships emerged. Increased snail size alone did not guarantee resistance. In adult snails, high numbers of spread cells ensured resistance, but similarly high counts in juvenile snails did not. When mRNA levels were compared in R and S lines of equally low spread cell number, *peroxiredoxin1* (*prx1*) and a gene for an inferred phagocyte oxidase (*phox*) subunit were twofold higher in R relative to S snails. The gene for allograft inflammatory factor (*aif*) was also expressed at higher levels in R snail lines, regardless of the numbers of spreading haemocytes characterizing the lines. The first two genes add further evidence of critical roles for ROS-dependent killing mechanisms in this host–parasite system, while *aif* suggests that constitutive activation of haemocytes may also contribute to the R phenotype.

2. Materials and methods

2.1. Animal care

Animals were handled following protocols approved by the Oregon State University, USA, Animal Care and Use Committee according to requirements outlined in the United States National Research Council Guide for the Care and Use of Laboratory Animals, 8th edition. Animal numbers were held to the minimum required for the research.

2.2. Inbreeding followed by resistance phenotyping

Inbred snail lines were developed from juveniles of our 13-16-R1 (Oregon State University) laboratory population of *B. glabrata*. The provenance of this strain was described earlier (Bonner et al., 2012). Snails were isolated prior to sexual maturity and allowed to mature and self-fertilise. Two more generations of offspring were subsequently isolated as juveniles for two more full rounds of inbreeding. Fourth generation siblings were used to establish tank colonies (inbred lines).

Inbred lines were maintained in 26 °C de-chlorinated water supplemented with shell hardener (480 μM $CaCO_3$, 82 μM NaCl, 58 μM $MgCO_3$, 13 μM KCl). Filters contained activated charcoal; crushed coral was added to enhance buffering capacity. To minimise the chances of stress-related effects, snails were sampled between 1 and 4 weeks after water change. The snails were fed washed green leaf lettuce ad libitum and kept in an environment with a 12 h light–dark cycle.

To score susceptibility, 12 snails were placed individually in wells with 1.5 mL of artificial spring water (Ulmer, 1970) and exposed for 2 h to five freshly hatched *S. mansoni* miracidia. The parasite eggs were isolated from hamster livers and miracidia were prepared as previously described (Stibbs et al., 1979). After exposure each snail group was maintained in a tank under dim light (~3 lx) (Steinauer and Bonner, 2012). Infection was confirmed by cercarial shed from individual snails exposed to bright light at 5, 7 and 9 weeks post-exposure. The entire procedure was repeated once and yielded consistent results.

2.3. Spread cell counts

Haemolymph was collected from snails of 5 and 12 mm diameter, and haemocytes that extended pseudopods were counted. To minimise stress, each snail was placed in a small dish containing tank water at 26 °C. Sterile balanced salt solution (CBSS; 48 mM NaCl, 2 mM KCl, 0.5 mM Na_2HPO_4, 0.6 mM $NAHCO_3$, 5.5 mM glucose, 2.9 mM trehalose; Chernin, 1963) was pre-warmed to 26 °C. Directly prior to bleeding, snail shells were cleaned with de-chlorinated water and cotton swabs, and wiped dry. Cardiac punctures were performed (Bayne et al., 1980) and only haemolymph which pooled on the shell was sampled. Haemolymph from each individual snail was placed on Parafilm™ and shell debris was allowed to settle for 30 s. The clean, upper portion of haemolymph was mixed 1:1 with CBSS and loaded on a Neubauer Improved haemacytometer. After incubation in a 26 °C humid chamber for 30 min, the cells with pseudopodia were counted in five 1 mm squares at 200× magnification. Cell numbers were calculated as cells/μL of haemolymph. Over a period of 6 months, 10 snails of 12 mm diameter and three snails of 5 mm diameter were sampled from each inbred line.