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REVIEW

Molluscan response to parasite: *Biomphalaria* and *Schistosoma mansoni* interaction

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Abstract

Digenetic trematodes use molluscs, almost always a Gastropoda, in their evolutive cycle, as intermediary hosts. The genus *Schistosoma*, with three main species that infect humans - *S. mansoni, S. japonicum*, and *S. haematobium* – shows a prevalence of 200 million patients in various countries worldwide, and 600 million people are still at risk of infection. *S. mansoni* is the most prevalent species, and *Biomphalaria* snails are its intermediary hosts. Although the campaigns of schistosomiasis control based on chemotherapy have reduced the morbidity and prevalence of this disease, transmission continues in almost all the areas submitted to intervention. One of the factors that has influence on the susceptibility of *Biomphalaria* to *S. mansoni* infection is ability of the host internal defense system (IDS) to recognize and destroy the parasite. In *Biomphalaria,* the IDS is composed of cellular elements named hemocytes that act jointly with soluble components present in hemolymph, which could affect directly the larvae, or act in the recognition of the parasite, and activation of hemocytes. The susceptibility level of the mollusc has been attributed to the hemocyte capacity of involving and destroying the parasite, and this will be the centre of interest of this review.

The study of *S. mansoni* and *Biomphalaria* interaction in resistant snail strains is important not only due to the academic-scientific value of this fascinating research area, but also to the potentially possible alternatives for the control of this endemia.

Key words: *Schistosoma mansoni* sporocysts*; Biomphalaria glabrata; Biomphalaria tenagophila*; circulating hemocytes; soluble factors of hemolymph

Introduction

Although the great majority of the living beings is represented by invertebrates, up to now the publications in mass dealing with the defense mechanisms against pathogens is practically restricted to interactions between pathogens of vertebrate animals. The invertebrate animals must necessarily reckon upon their defense system to recognize and destroy infectious agents, although this system are not able to generate the diversity of recognition observed during the adaptative immune response of vertebrates (van der Knaap and Loker, 1990; Loker *et al.*, 2004).

Recent studies have demonstrated many similarities between the innate defense response of vertebrates and the internal defense system of invertebrates (Hoffman *et al.*, 1999; Hoffman, 2003), being identified in various invertebrates organisms production of complement-like proteins, anti-microbial peptides, pattern-recognition receptors (PRRs) such as toll-like receptor and C-type lectins, phagocytic cells, production of highly toxic metabolites of oxygen and nitrogen (Loker *et al.*, 2004). Although many similarities were identified in the defense system of several groups of invertebrates, it is important to note that genomic studies have indicated varied defense mechanisms in invertebrate groups phylogenetically associated,

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but with very different alimentary habits and habitat (Loker *et al.*, 2004).

Several groups of invertebrates, such as insects and molluscs, are important intermediary hosts of parasites species that are transmitted to humans and domestic animals. As an example we can mention the necessity of Diptera insects genus *Lutzomya* in the development of different species of *Leishmania*, the anophelins in the transmission of Malaria or the anophelins and culicides for the development of lymphatic filariosis. Among the molluscs, Gastropoda are obligatorily intermediary hosts in the development of the majority of digenetic trematode species, such as *S. mansoni* and *Fasciola hepatica.* Thus, it is of the utmost importance to get a better understanding of the effector mechanisms used by the internal defense system (IDS) of these invertebrates for the development of new strategies related to the control of these parasite infections. These studies have also contributed significantly to a better knowledge about the innate response of vertebrates. This review will focus primarily on the response of *Biomphalaria* during infection with *S. mansoni*.

Schistosoma mansoni infection in *Biomphalaria*

Schistosomiasis is an important health problem that affects over 200 million people worldwide. Among the schistosomes species that infect human beings, *S. mansoni* is transmitted by *Biomphalaria* snails and causes intestinal and hepatic schistosomiasis in Africa, Arabian Peninsula, and South America (Gryseels *et al.*, 2006). Recent estimative indicates that 6-7 million people are infected by *S. mansoni* only in Brazil (Katz and Peixoto, 2000).

S. mansoni infects *Biomphalaria* by means of active penetration of the parasite´s ciliated larvae, named miracidia, at any site of the snail's exposed parts, frequently the base of the antennae and cephalopodal mass. In the process of penetration, the parasite undergoes morphological and physiological changes, being transformed into primary sporocyst that remains in the fibro-muscular tissue of the host´s cephalopodal region near the penetration site. The primary sporocysts generate the secondary ones, which migrate from the cephalopodal musculature to the digestive glands or hepatopancreas of the mollusc, where they undergo profound anatomic changes and their germinative cells can generate the cercariae (Maldonado and Acosta-Matienzo, 1947; Pan, 1965; Pereira *et al.*, 1984).

In Brazil, out of ten species of molluscs genus *Biomphalaria* described, only three were found naturally infected by *S. mansoni: B. glabrata, B. tenagophila* and *B. straminea* (Paraense, 2001). The susceptibility level of these different species of *Biomphalaria* to infection with the same lineage of *S. mansoni* is much diversified, and *B. glabrata* may present up to 75.3 % of susceptibility in experimental infections, *B. tenagophila* 32.6 %, and *B. straminea* 11.3 %, as demonstrated by Souza *et al.* (1997). Besides the difference in susceptibility observed among *Biomphalaria* species compatible with the parasite, some lineages or geographic isolates of a same species of *Biomphalaria* also present a great variation of susceptibility to the parasite. As far as *B. tenagophila* is concerned, the geographic lineage isolated at the Biological Reservoir in Taim (Rio Grande do Sul, Brazil) was found to be completely resistant to the development of all *S. mansoni* isolates already tested. The character of resistance of this *B. tenagophila* lineage has been explored at our laboratory, aiming at studying the possible mechanisms of the parasite's destruction, representing a potential model for the control of transmission in endemic areas, where *B. tenagophila* is the unique transmitter agent of the disease (Coelho *et al.*, 2004).

Genetic control of resistance of *Biomphalaria* to *S. mansoni* infection

The compatibility between *S. mansoni* and its intermediary host is influenced by behavioral and physiological factors of the mollusc. Once found a suitable host, the susceptibility level of *Biomphalaria* to *S. mansoni* can be determined by the genetic differences of the molluscs, as well as by the genetic constitution of *Schistosoma* (Basch, 1976). Newton (1952, 1953) demonstrated for the first time that the susceptibility of *B. glabrata* snail to *S. mansoni* depends largely upon genetic factors. Later, these results were corroborated by Richards (1970), who demonstrated that the resistance character, acquired at the maturity phase, is determined by a single dominant gene, with mendelian inheritance. Nevertheless, in *B. glabrata*, age is a determinant factor of the snail susceptibility; juvenile snails being more susceptible to infection even in lineages where the adult snail is resistant to *S. mansoni* infection. Thus, the susceptibility of juvenile *B. glabrata* to *S. mansoni* infection is also regulated by genetic factors, being estimated that four or more genes affect this character (Richards, 1977).

B. tenagophila Taim is a lineage that presents absolute resistance to infection by all *S. mansoni* strains tested at any phase of the mollusc development (Santos *et al.*, 1979; Martins-Souza *et al.*, 2003; Coelho *et al.*, 2004). Crossbreedings between *B. tenagophila* Taim and *B. tenagophila* BH (susceptible lineage) showed that the character of resistance to *S. mansoni* infection is dominant (Santos *et al.*, 1979). The dominance of the resistance character of *B. tenagophila* Taim was confirmed in crossbreedings with the susceptible Joinville lineage (Rosa *et al.*, 2005), showing that 100 % of the F_1 offspring were resistant and only 8 % of the F_2 offspring were susceptible to infection by the parasite (Table 1). This study suggests that at least two dominant genes would be responsible for the resistance to *S. mansoni* observed in *B. tenagophila* Taim, one of them being the most important since it was expressed in all F_1 offspring (Rosa *et al.*, 2005). The dominance of the resistance character in *B. tenagophila* Taim added to the reproductive success of this lineage in the presence of susceptible snails of the same species (Rosa *et al*., 2006), led us to suggest the use of this lineage as an alternative for biological control in

Table 1 Susceptibility rates of *B. tenagophila* Joinville, *B. tenagophila* Taim, F1 and F2 when submitted to infection with 25 miracidia of LE strain of *S. mansoni*

Experiment	Group	Number of snails exposed to *S. mansoni*	Number of surviving snails	Number of infected snails eliminanting cercariae (%)
1	Taim	64	60	0 (0)
	Joinvillle	35	12	7 (58.3)
	F1	170	150	1 (0.6)
	F2	110	87	7 (8)
2	Taim	30	25	0 (0)
	Joinville	30	15	9 (60)
	F1	50	44	0 (0)
	F2	50	38	2 (5.3)

some areas of schistosome transmission (Coelho *et al.*, 2004).

One of the factors that influence the susceptibility and may be genetically determined is the activity of the snails IDS. Experimental infections in *B. tenagophila* Taim have shown that *S. mansoni* miracidia are able to penetrate this snail lineage, however the parasites induce an intense cellular infiltration and are rapidly destroyed, suggesting an important participation of the IDS on determination of resistance to *S. mansoni* in *B. tenagophila* Taim.

Internal defense system (IDS) of the mollusc

The IDS of snails is composed of cellular elements constituted by hemocytes, and by soluble factors present in hemolymph. The hemocytes may be circulating in hemolymph or fixed in tissues. In Planorbids the hemolymph circulates in a semi-open system impelled by the heart. The hemoymph leaves the heart through the aorta reaching the tissues, draining in the venous sinus and returning to the heart via the pulmonary and renal veins, after being re-oxygenated in the pulmonar wall (Baker, 1945). The heart, enclosed by the pericardium membrane, is divided into two chambers, the auricula, which receives hemolymph from the pulmonary cavity, and the ventricule that impels the hemolymph through the aorta. The aorta is divided into two arteries: the visceral artery, which irrigates the posterior part of the snail´s body, including the digestive and genital systems, and the cephalic artery, that reachs all the cephalopodal region. The arteries are exhausted in the pseudovascular spaces of the tissues, accumulating hemolymph in three venous sinuses: cephalopodal, visceral and sub-renal, returning to the heart after circulating through the kidney and lung (Baker, 1945; Paraense, 2001).

In *B. glabrata* and *Bulinus sp* a well defined region, located between the pericardium and the posterior epithelium of the mantle cavity (Fig. 1A), also called amebocyte producing organ (APO), was identified as the main site for the production of hemocytes (Lie, 1976). Recent observations (Sullivan *et al.*, 2004; Sullivan and Castro, 2005) showed an increase of mitoses in the cells of this region, ranging from 24-72 h after inoculation of antigens of *S. mansoni* miracidia or cercariae, this being more evident in resistant lineage of *B. glabrata*. Nevertheless, some authors (Matricon-Gondran, 1990; Souza and Andrade, 2006) demonstrated that *B. glabrata* hemocytes may present multi-centric origin, and sites with proliferation of hemocytes were detected also at the saccular portion of the renal tubules and in the ventricular cavity of the heart (Fig. 1B).

The circulating hemocytes of different species of molluscs present morphological and functional heterogeneity. According to Ottaviani (1992; 2006), the population of circulating hemocytes of the majority of gastropod molluscs is constituted by two cellular types: the starry hemocytes that emit pseudopodes, and the roundish hemocytes. In *Planorbarius corneus*, the starry hemocytes are cells that present phagocytic activity, adhere to glass and express proteins that are recognized by pro-inflammatory anti-cytokine antibodies of vertebrates. On the other hand, the roundish hemocytes are not endowed with phagocytic activity, they are not able to adhere to glass, and besides they proliferate in the presence of phytoagglutinin (Ottaviani, 1992; Ottaviani *et al.*, 1993). Similarly to *P. corneus*, the majority of the authors (Harris, 1975; Lie *et al.*, 1987; Barraco *et al.*, 1993; Borges and Andrade, 2003) also distinguish two sub-populations of circulating hemocytes in hemolymph of *B. glabrata.* These sub-populations are called granulocytes, *i.e.*, the hemocytes that emit pseudopodes and produce phagocytosis, and hyalinocytes that are the small and roundish hemocytes (Fig. 2A). Granulocytes can be easily identified by the uptake of neutral red stain into the cell vesicles (Fig. 2B), showing that *S. mansoni* infection induce cellular proliferation (Martins-Souza *et al.*, 2003). However, ultra-structural studies (Matricon-Gondran and Letorcart, 1999 a,b), analyses of distribution and abundance of lysosomal enzymes (Granath and Yoshino, 1983), as well as of expression of lectin-ligants on the cellular surface (Joky *et al.*, 1983; Martins-Souza *et al.*, 2006) suggest that the circulating hemocytes of *Biomphalaria* constitute a cellular population significantly more heterogenous than that previously described (Fig. 2B). The phenotypical and functional definition of *Biomphalaria* hemocytes is of fundamental importance to understand the participation of these cells, or of any cellular sub-population, in the destruction mechanism of *S. mansoni* larvae or other parasites.

Even though hemocytes are the main component of the mollusc IDS, there are some experimental

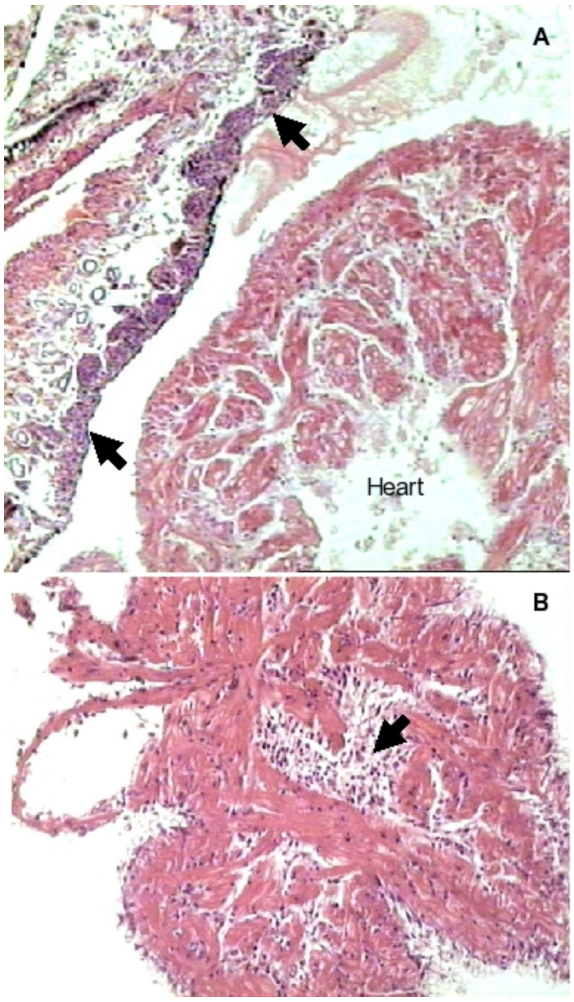

Fig. 1 Photomicrographs of *Biomphalaria glabrata* heart region. **(A)** Snail heart tissue in close contact with the pericardial membrane. The arrows indicate the amebocyte-forming organ (APO), a narrow and long band of epithelial-like cell along the pericardial membrane (100x). **(B)** Heart tissue with a dense collection of hemocytes (Arrow) (200x). Hematoxylin-Eosin stain

evidences indicating that soluble elements of the hemolymph would participate in the protective mechanism against pathogens. Soluble components of the hemolymph of molluscs can directly interact with pathogenic agents, by means of production of toxic substances or lytic peptides, or indirectly through mediator molecules for recognition of the pathogen or hemocyte activators. Peptides with anti-microbial function, called mytilines, are produced and stored in hemocyte granules, and they are secreted in hemolymph of *Mytilus galloprovincialis* (Mitta *et al.*, 2000) notwithstanding the participation of these peptides in the destruction of bacterial infections, there are no evidences of the participation of these peptides in the interaction of mollucs with metazoan parasites.

Fryer and Bayne (1996) show that particles of polystireno treated with soluble factors of hemolymph of *B. glabrata* are significantly more phagocyted by hemocytes than the untreated particles, demonstrating that soluble factors of hemolymph may participate of the recognition mechanism and opsonization of particles by hemocytes. Johnston and Yoshino (1996) demonstrate that lectins similar to those of *Conavalia ensiformis* (ConA), *Erythrina corallodendrom* (ECA), *Glycine max* (SBA), *Tetragonolobus purpureas* (TPA), and *Triticum vulgaris* (WGA) are present in hemolymph of *B. glabrata*. In molluscs, lectins are synthesized by hemocytes and released in hemolymph, where they immobilize the material particularized by agglutination, or are expressed at the surface of circulating hemocytes, where apparently they act as cytophylic receptors (Richards and Renwrantz, 1991; Fryer and Bayne, 1989).

Besides the lectins, other proteins with homologous function to cellular mediators, and already characterized in vertebrates, have been identified in hemolymph of molluscs and may be involved in the activation of hemocytes during infection by digenetic trematodes (Ottaviani *et al.*, 1993, 1995). Ottaviani and co-workers (1993) reported the presence of a variety of proteins similar to pro-inflammatory cytokines of vertebrates, including interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), and alpha Tumoral Necrosis Factor (TNF-α) in hemocytes of two species of molluscs, *Planorbarius corneus* and *Viviparus ater,* being present only in hemocytes with phagocytic activity. In further studies, Ottaviani *et al.* (1995), relate the production of homologous proteins to cytokine with an increase of phagocytic activity and induction of nitric oxide synthase (NOS) of mollusc hemocytes. These results suggest that cytokines-like may participate in the activation of hemocytes.

Functional mechanisms in parasite-mollusc interaction

In the last years, many aspects of the interaction between the digenetic trematode larvae and the internal defense system of molluscs have been elucidated. Nevertheless, the possible mechanisms responsible for destruction of the majority of larvae in resistant snails remain to be totally understood. The results reported up to now suggest that the hemocyte could be the effector element in the destruction mechanism of trematodes, being directly involved in the death of some encapsulated parasites (van der Knaap and Loker, 1990; Bayne *et al.*, 2001) or in the production of soluble factors which could be cytotoxic (Connors *et al.*, 1995). The majority of the authors (Connors *et al.*, 1995; Bayne *et al.*, 2001; Martins-Souza, 2003) agree that the snails´ defense generally occurs by means of destruction, total or partial, of the primary sporocyst at the first few hours following the penetration of the miracidium.

The existence of a cellular defense mechanism deployed by molluscs against trematode infection was initially suggested by the finding of histological reactions around parasite sporocysts (Newton, 1952). Further studies have shown that hemocytes infiltration around parasite larvae in *S. mansoni*-

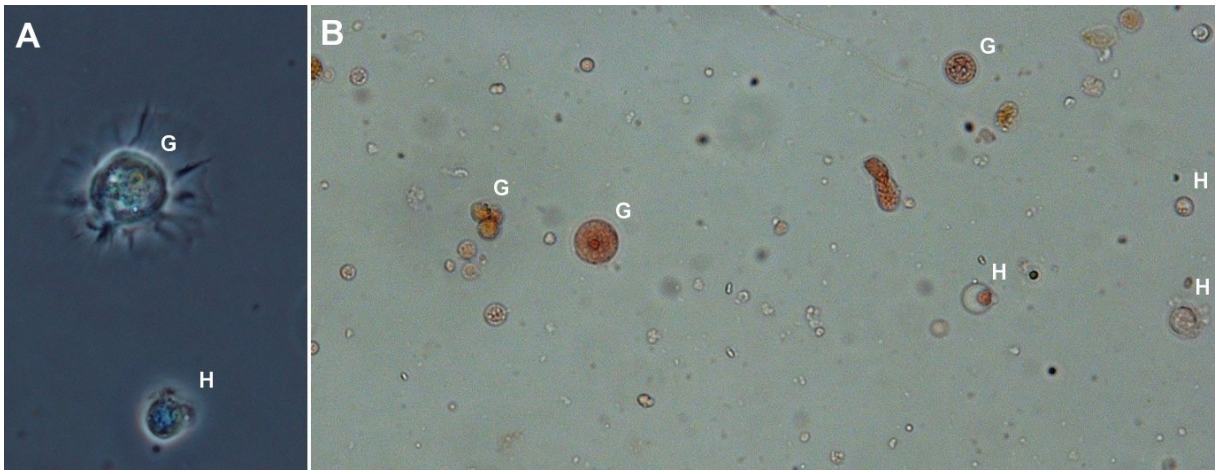

Fig. 2 Morphological aspects of circulating hemocytes from *Biomphalaria tenagophila*. (**A**) Phase contrast photomicrography showing a granulocyte (G) and a hyalinocytes circulating in *B. tenagophila* hemolymph (400x). (**B**) Bright field photomicrography of *B. tenagophila* circulating hemocytes after addition of neutral red staining, showing the heterogeneous granulocyte population stained in red (G) and the non-stained cells designated as hyalinocytes (H) (200x)

infected *Biomphalaria* was stronger in snail species that are more resistant to parasite infection, such as *B. tenagophila* and *B. straminea* (Souza *et al*., 1997). In highly susceptible *B. glabrata*, confirmed by the great quantity of cercariae eliminated during a long period of time, sporocysts and cercariae at different developmental stages are found in abundance in the inner of the host, resulting in compression of the host´s structures, mainly in the interstice of the digestive glands, ovotestis and renal tubules. However, the presence of a great number of parasites did not induce cellular reaction in the parasite susceptible snail strains (Godoy *et al*., 1997). On the opposite extreme appear the resistant snails, which shed a few cercariae and show an extensive infiltration in tissues with numerous hemocytes, frequently placed around the parasite structures in disintegration. The focal reactions frequently assumed a granuloma-like appearance (Godoy *et al*., 1997). In our experimental model, *S. mansoni* infection in *B. tenagophila* of Taim strain resulted in intense, diffuse or granuloma-like cellular infiltration in the infection site, mainly in the connective tissue of the snail cephalopodal region and antennae (Fig. 3). The cellular infiltration was detected few hours after the parasite infection and no viable sporocysts were recovered from these infected snails. Direct evidence of the hemocyte participation in *S. mansoni* infection control was obtained with experiments that transferred the APO from resistant to susceptible snail strains. In *B. glabrata*, the transplantation of APO from miracidia-exposed resistant strain to susceptible NIH snails resulted in significantly more killed sporocysts than as observed during *S. mansoni* infection in NIH snails (Sullivan and Spencer, 1994). Recently, Barbosa *et al.* (2006) showed in *B. tenagophila* that transplantation of the hematopoietic organ from Taim lineage (totally resistant) to a susceptible *S. mansoni* strain resulted in an absolute resistance in the receptors whose transplant was successful. Moreover, inoculation of silica particles in *B. tenagophila* Cabo Frio resulted in transitory reduction of a macrophage-like cell population of circulating hemocytes. The cellular depletion induced by silica-treatment in *B. tenagophila* Cabo Frio was accomplished by enhanced susceptibility to *S. mansoni* infection, shortening the intramolluscan phase of the parasite and increasing the number of sporocysts and cercariae produced (Martins-Souza *et al.*, 2003).

The process of destruction of *S. mansoni* larvae by hemocytes initiates with the recognition and encapsulation of the newly-penetrated sporocyst. Bayne and co-workers (1980b) demonstrated that cell-free hemolymph obtained from susceptible and resistant *B. glabrata* strains are unable to change visibly the morphology of *S. mansoni in vitro,* the same occurring with hemolymph containing hemocytes of susceptible lineages. However, hemocytes of susceptible strains associated with soluble factors of hemolymph of resistant *B. glabrata* lineages acquire the ability to destroy *S. mansoni* sporocysts. The importance of the soluble fraction of *Biomphalaria* hemolymph in the destruction mechanism of *S. mansoni* sporocysts was also confirmed *in vivo* in studies dealing with the transference of this fraction obtained from resistant *B. glabrata* snails to other ones susceptible to the parasite (Granath and Yoshino, 1984). Recent results obtained with *B. tenagophila* Taim also showed that addition of the cell-free hemolymph of this resistant snail strain significantly increased the ability of hemocytes from susceptible strain of *B. tenagophila* (Cabo Frio or Joinville) to destroy *S. mansoni* sporocysts, *in vitro*. Moreover, the increased mortality of sporocysts was associated with higher number of hemocytes on the parasite tegument (Fig. 4), suggesting that cell-free hemolymph from resistant snail strain increased parasite recognition by hemocytes. However, in contrast with *B. glabrata* (Bayne *et al.*, 1980), cell-free

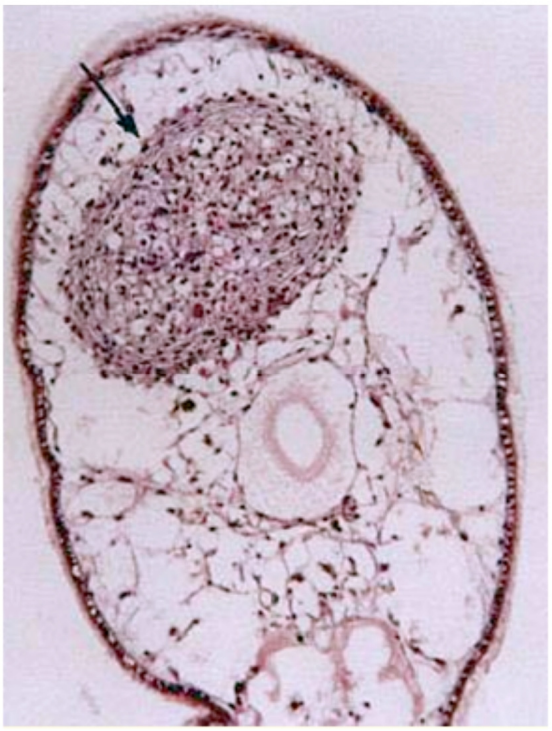

Fig. 3 Intense hemocyte infiltration in *Schistosoma mansoni* infected *Biomphalaria tenagophila* Taim, a parasite-resistant snail strain. Photomicrograph of a transversal section of antennae tissue from *B. tenagophila* Taim 15 days after *S. mansoni* infection (20 miracidium/snail), showing a focal cellular reaction (arrow) with a granuloma-like appearance (200x). Diffuse and focal cellular infiltration is observed in cephalopodal tissue of parasite infected resistant snails and has been associated with the parasite penetration and destruction. Hematoxylin-Eosin stain

hemolymph from *B. tenagophila* Taim was able to destroy a small, but statistically significant, percentage of *S. mansoni* sporocysts even in absence of hemocytes (Pereira, 2005). The importance of *B. tenagophila* Taim cell-free hemolymph in *S. mansoni* control was also confirmed *in vivo*, since susceptible snails treated with *B. tenagophila* Taim cell-free hemolymph had lower percentage of infectivity (Pereira, 2005; Coelho and Bezerra, 2006), and the snails that got infection produced lower number of sporocysts and cercariae (Pereira, 2005).

The main components of *S. mansoni* sporocysts tegument is glicoproteins and glicolipides (Zelck and Becker, 1990; Uchikawa and Loker, 1991). Johnston and Yoshino (1996) showed that lectins from *B. glabrata* cell-free hemolymph bind to glicoproteins extracted from the parasite tegument. More recently (Adema *et al.*, 1997a,b) a group of proteins, homologous to fibrogen and that has been associated with recognition, was identified in *B. glabrata* hemolymph, being its expression enhanced after infection of the mollusc with *Echinostoma paraensei,* another digenetic trematode. These results suggested that lectins would serve as cell surface receptors for carbohydrate structures from trematode parasites. Moreover, soluble lectins would also participate in the recognition mechanism by binding to carbohydrate structures from both hemocytes and parasite tegument (van der Knapp *et al.*, 1990).

Besides participating in the recognition of *S. mansoni*, lectins can also activate hemocytes. Hemocytes of susceptible and resistant *B. glabrata* strains were stimulated with bovine albumin associated with one of the six carbohydrates: mannose, galactose, fucose, N-acetyl-glucosamine, N-acetyl-galactosamine and lactose, that are present in the tegument of *S. mansoni* sporocysts. Hemocytes stimulated with BSA-galactose, BSA-mannose, and BSA-fructose were able to produce reactive oxygen-species (ROS) (Hahn *et al.*, 2000).

Our results also confirmed the participation of soluble lectins in *S. mansoni*-sporocysts recognition mechanisms by *B. tenagophila* species. Circulating hemocytes recovered from *B. tenagophila* - both Taim and Cabo Frio strains - were intensively labelled by FITC-conjugated lectins, such as PNA, SBA, and WGA. Moreover, *S. mansoni* infection in resistant snail strain (Taim) resulted in initial reduction in number of labelled-hemocytes in circulation (Martins-Souza *et al.*, 2006). The reduction of circulating hemocytes during the first few hours after *S. mansoni* infection has been associated with the cell recruitment to the infection site (Bezerra *et al.*, 1997; Martins-Souza, 2003).

Other proteins similar to pro-inflammatory cytokines of vertebrates have been identified in hemolymph of molluscs and can participate in activation of hemocytes during the destruction process of parasites. Specifically in *B. glabrata* was identified a protein with immunological and functional similarity to interleukin-1-like (IL-1 like). In this snail, IL1-like protein is induced by *S. mansoni* infection, and the level was significantly higher in resistant snail strains (Granath *et al.*, 1994). The inoculation of human recombinant IL-1α in susceptible strain of *B. glabrata* resulted in increased production of ROS by circulating hemocytes and reduced number of *S. mansoni* cercariae upon parasite infection (Connors *et al.*, 1995). These authors confirmed, *in vitro*, that cell-free hemolymph recovered from rhIL-1α-treated snail, but not only rhIL-1α, was capable of destroy *S. mansoni* sporocysts, suggesting that IL-1 would activate *B. glabrata*-hemocytes to produce and secrete soluble cytotoxic mediators (Connors *et al.*, 1998).

The effector mechanisms by which activated hemocytes are able to kill trematode larvae are not fully understood yet. Dikheboom *et al.* (1988a,b) showed for the first time that gastropod hemocytes do produce reactive oxygen species (ROS) in response to trematode infection. The initial reduction of O_2 to superoxide anion (O_2) is catalyzed by NADPH-oxidase and O_2 can be converted to other ROS, including hydrogen peroxide (H_20_2) and hypochlorous acid (HOCI) (Hampton *et al.*, 1998; Bayne *et al.*, 2001). NADPH oxidase-like activity has been identified in gastropod hemocytes (Adema *et al.*, 1993) and ROS production

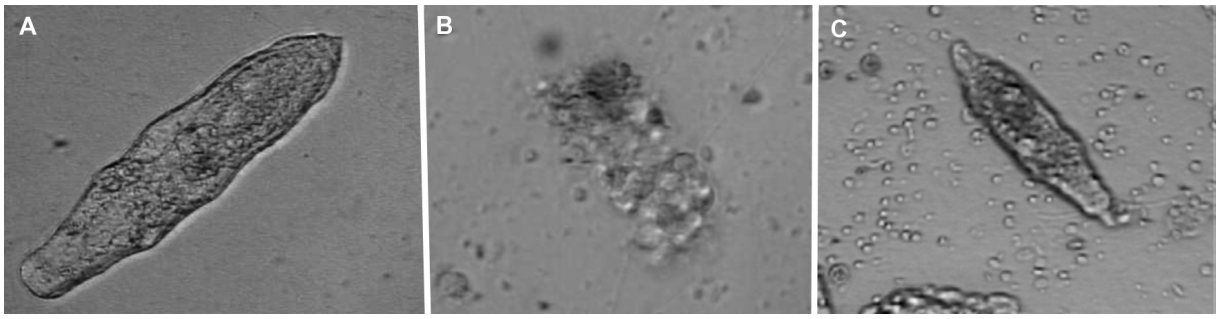

Fig. 4 Interaction of *Schistosoma mansoni* sporocyst and hemocytes from *Biomphalaria tenagophila* Taim (**B**) or *Biomphalaria glabrata* BH (**C**). (**A**) *S. mansoni* sporocyst in Chernin's Balanced Salt Solution, showing an intact parasite larva (200x). (**B**) *S. mansoni* sporocyst completely encapsulated by hemocytes from *B. tenagophila* Taim, a snail strain totally resistant to parasite infection (100x). (**C**) *S. mansoni* sporocyst incubated with hemocytes from *B. glabrata* BH, snail highly susceptible to parasite infection, showing an intact parasite with very few cell attached, x100

by *B. glabrata* hemocytes has been associated with *S. mansoni* resistance (Adema *et al.*, 1994). Additionally, molluscan hemocytes also generate nitric oxide (NO) from molecular oxygen and arginine (Conte and Ottaviani, 1995; Arumugan *et al.*, 2000). Experimental evidences of ROS and/or NOS participation in killing of *S. mansoni* sporocysts by *B. glabrata* hemocytes were obtained using specific oxidant scavengers or enzyme inhibitors during the *in vitro* association. The results showed that inhibition of H_2O_2 and NO production do favor sporocysts survival, indicating that this reactive species would be toxic to trematode larvae (Hahn *et al.*, 2001 a,b). However, attempts to associate H_2O_2 or NO production with snail strain susceptibility to *S. mansoni* infection have not been conclusive (Hahn *et al.*, 2000; Bayne *et al.*, 2001). Similarly, we were able to estimate NO production by hemocytes isolated from *B. glabrata* or *B. tenagophila* at different time after *S. mansoni* infection and re-stimulated *in vitro* with *S. mansoni* egg antigen (SEA). At each experimental point, as well as for each snail strain, analyses were performed in triplicate with total hemolymph collected and gathered together from 3 snails. After centrifugation, the hemocyte pellet was ressuspended in Chernin´s Balanced Salt Solution (5 x 10^5/ml of CBSS) containing 100 μg/ml of SEA, plated in 96-well tissue culture plates and incubated at 26 °C and 5 % CO_2. After 18 h of incubation, NO presence was assayed directly in the cell supernatant by the Greiss reaction (Green *et al.*, 1982) that quantifies the nitrite contents of the supernatants, as detailed by Perreira *et al.* (2006). The NO level increased in hemocytes recovered after the first few days of *S. mansoni* infection, however there were no detectable differences in NO level between the snails, although each species or strain shows remarkable difference in parasite susceptibility (Fig. 5). However, it is important to confirm if supernatant level do reflect the local production in hemocyte-sporocyts interaction. Besides producing reactive species of oxygen and nitrogen, microscopy analyses indicate that hemocytes from parasite-resistant snails would phagocvte portions of sporocyst tegument leading to mechanical lesion that may be also lethal to the parasite (van der Knaap and Loker, 1990).

Finally, in order to evaluate the efficiency of *Biomphalaria spp* defense system in the destruction of *S. mansoni* larvae, one must consider that there are many evidences indicating that the parasite is able to develop strategies to allow its evasion. Thus, it has been described that the primary sporocyst of *S. mansoni* acquires quickly the antigens present in the host´s hemolymph (Bayne *et al.*, 1986), as well as express in the tegument antigens similar to those expressed by the host´s cells (Yoshino and Bayne, 1983) hindering the recognition process of the parasite by hemocytes. It has been also reported that components of the excreted/secreted material by the miracidium during the transformation process may reduce the motility of hemocytes, as well as their phagocytic capacity (Connors and Yoshino, 1990; Lodes and Yoshino, 1990), thus justifying the cellular reaction almost inexistent observed around *S. mansoni* sporocysts present in the tissue of *B. glabrata* susceptible strains.

In addition to avoid the hemocytes´ approach, it has been also reported that sporocysts incubated *in vitro* with hemocytes of susceptible strains may be encapsulated, but not destroyed (Boehmler *et al.*, 1996; Hahn *et al.*, 2001a), suggesting the existence of anti-oxidant mechanisms.

Perspectives

The most recent studies related to the internal defense system of invertebrates have shown new aspects of the invertebrate-pathogen relationship. These aspects afforded us a better understanding of the recognition and cellular activation mechanisms, which are also present in the innate defense response of vertebrates. *Biomphalaria-Schistosoma mansoni* interaction, besides being an important model in human health, constitutes an experimental approach that may add important information to the knowledge of the defense mechanism utilized by invertebrates, as well as of phylogenetic evolution of these mechanisms.

In this context, our group has carried out researches dealing with the phenotypic and functional

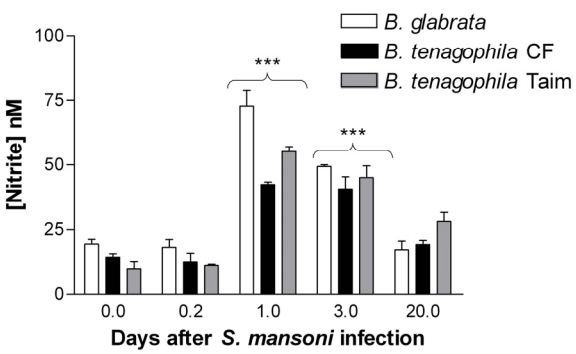

Fig. 5 Nitric oxide (NO) level in supernatant of circulanting hemocytes recovered from *Biomphalaria glabrata*, *B. tenagophila* Cabo Frio e *B. tenagophila* Taim during the *S. mansoni* infection. NO levels was indirectly estimated by quantification of nitrite, using the Greiss reaction, in hemocyte supernatant recovered after 18 h of *in vitro* re-stimulation with soluble egg antigen (SEA 100 μg/ml). *** for $P < 0.001$ when comparing with nitrite level obtained after stimulation of non-infected hemocytes from the same snail strain. At each time point, there was no statistically significant difference in the nitrite level between the snail strains

characterization of the hemocytes and hemolymph from different strains of *B. glabrata* and *B. tenagophila.* Our goal is to identify possible mechanism of trematode recognition and hemocyte activation in *B. tenagophila* Taim that would be responsible to the fast parasite destruction and consequent resistance against *S. mansoni* infection observed in this snail strain. In parallel, we are investigating the heritage of the resistance character from *B. tenagophila* Taim to the offspring resulted of crossbreeding with the susceptible snail strains. A more comprehensive identification of these mechanisms would expand the theorical base that gives support to mass introduction of *B. tenagophila* resistant strain from Taim in areas where transmission is maintained by this species (Coelho *et al.*, 2004).

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