

Differential lectin labelling of circulating hemocytes from *Biomphalaria glabrata* and *Biomphalaria tenagophila* resistant or susceptible to *Schistosoma mansoni* infection

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Lectins/carbohydrate binding can be involved in the Schistosoma mansoni recognition and activation of the Biomphalaria hemocytes. Therefore, expression of lectin ligands on Biomphalaria hemocytes would be associated with snail resistance against S. mansoni infection. To test this hypothesis, circulating hemocytes were isolated from B. glabrata BH (snail strain highly susceptible to S. mansoni), B. tenagophila Cabo Frio (moderate susceptibility), and B. tenagophila Taim (completely resistant strains), labelled with FITC conjugated lectins (ConA, PNA, SBA, and WGA) and analyzed under fluorescence microscopy. The results demonstrated that although lectin-labelled hemocytes were detected in hemolymph of all snail species tested, circulating hemocytes from both strains of B. tenagophila showed a larger number of lectin-labelled cells than B. glabrata. Moreover, most of circulating hemocytes of B. tenagophila were intensively labelled by lectins PNA-FITC and WGA-FITC, while in B. glabrata small hemocytes were labeled mainly by ConA. Upon S. mansoni infection, lectin-labelled hemocytes almost disappeared from the hemolymph of Taim and accumulated in B. glabrata BH. The role of lectins/carbohydrate binding in resistance of B. tenagophila infection to S. mansoni is still not fully understood, but the data suggest that there may be a correlation to its presence with susceptibility or resistance to the parasite.

Key words: *Schistosoma mansoni* - *Biomphalaria tenagophila* - snail susceptibility to trematode - lectin labelling of hemocytes - host-parasite interaction

Some snail species of the genus *Biomphalaria* are obligatory hosts for the development of the larval stages of *Schistosoma mansoni* life cycle. The capacity of the inner defense system (IDS) of that host to destroy the parasite is one of the factors that may determinate *Biomphalaria* susceptibility to *S. mansoni* infection (Souza et al. 1997, Yoshino et al. 2001, Martins-Souza et al. 2003). The IDS of *Biomphalaria* remains not completely understood, but it is well known that it is composed of cellular elements named hemocytes, and soluble components that could directly affect the larvae and/or act on the recognition of the parasite and activation of hemocytes.

Hemocytes are the main mediators of the defense system of molluscs, taking part in phagocytosis of particles (e.g. bacteria and protozoans), as well as in encapsulation of larger parasites, as for example helminthic larvae (van der Knaap & Loker 1990). Nevertheless, participation of the soluble fraction of *B. glabrata* hemolymph (Granath & Yoshino 1984) or of *B. tenagophila* (Pereira 2005) in the mechanism of destruction of *S. mansoni* sporocysts is also important. This was confirmed in vivo by the increase of resistance to the parasite in susceptible snails treated with hemolymph of resistant ones.

The presence of substances in *B. glabrata* hemolymph that promote agglutination of hemocytes surrounding *S. mansoni* sporocysts was reported by Loker et al. (1984). Afterwards, Fryer and Bayne (1996) demonstrated that particles of polystyrene treated with soluble factors of hemolymph from resistant strains of *B. glabrata* are more significantly phagocytized by hemocytes from susceptible strains than the untreated particles, thus suggesting that soluble factors of hemolymph participate in the recognition mechanism and opsonization of particles by hemocytes.

In the search for possible mediators related to the parasite's recognition mechanism, it was demonstrated that purified lectins of *Conavalia ensiformis* (ConA), *Erythrina corallodendrom* (ECA), *Glycine max* (SBA), *Tetragonolobus purpurea* (TPA), and *Triticum vulgare*, among others, were able to bind to proteins present on the surface of *S. mansoni* sporocysts (Yoshino 1976, Uchikawa & Loker 1991, Johnston & Yoshino, 1996). Johnston and Yoshino (1996) showed that proteins from the soluble fraction of *B. glabrata* hemolymph were also capable of binding to the majority of sporocyst proteins recognized by purified lectins. Binding of these hemolymph proteins was strongly inhibited when sporocyst proteins were treated with periodate, suggesting participation of carbohydrates in this interaction. Thus, lectins could functionally intermediate binding of hemocytes to the larval tegument of trematodes (van der Knaap & Loker 1990). Moreover, the binding lectin-carbohydrate possibly leads to a structural change of the complex, that could induce hemocyte activation (Bayne 1990), resulting in an

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increase of the phagocitary activity (Fryer et al. 1989) and/or of production of reactive oxygen derivatives (ROI) (Hahn et al. 2000). For this reason, the resistance or susceptibility of species or strains of *Biomphalaria* to *S. mansoni* infection could be related to qualitative or quantitative differences in the production of some lectins (Zelck & Becker 1990). Nevertheless, various experiments showed no difference in lectin composition of hemolymph from susceptible and resistant strains of *Biomphalaria* (Johnston & Yoshino 1996).

In molluscs, lectins are synthesized by hemocytes and released in hemolymph, or are expressed on the surface of circulating hemocytes, where they are able to act as cytophylic receptors (Richards & Renwranz 1991). Thus, another possibility that has been poorly explored in the literature is that differences in the recognition of the parasite could be related to differences in the expression of lectin ligands in hemocytes of different snail strains. Therefore, the objective of this experimental study was to verify lectin binding on circulating hemocytes from *B. glabrata* BH, a snail strain that is highly susceptible to *S. mansoni* infection (Paraense & Corrêa 1963), as well as in hemocytes from *B. tenagophila* Cabo Frio, snail strain with moderate susceptibility to *S. mansoni*, and *B. tenagophila* Taim that is completely resistant to the parasite infection (Martins-Souza et al. 2003). Moreover, we intend to verify if the resistance of *B. tenagophila* Taim to *S. mansoni* would be associated with the variation of expression of lectin-hapten on the circulating hemocytes.

MATERIALS AND METHODS

Parasite - The snails above mentioned were infected with *S. mansoni* of LE strain (isolated from a patient at Belo Horizonte, MG, Brazil). This strain has been maintained at the laboratory of the Schistosomiasis Research Unit, Federal University of Minas Gerais, Brazil, for 35 years.

Snails - Snail species of *B. glabrata* and *B. tenagophila* used in this study has been bred and maintained according to the procedures previously described by Pellegrino and Katz (1968) at the mollusc room of the Schistosomiasis Research Unit, Institute of Biological Sciences, Federal University of Minas Gerais, Brazil, for at least 25 years. The lineage of *B. glabrata* utilized and named BH strain proceeds from the Pampulha lake, Belo Horizonte, MG, Brazil. This snail strain is highly susceptible to the infection to *S. mansoni* (Paraense & Corrêa 1963). Two strains of *B. tenagophila* were selected for the study. The Taim strain collected at the Ecological Station of Taim, Rio Grande do Sul, Brazil, and the Cabo Frio strain collected at Cabo Frio, Rio de Janeiro, Brazil. *B. tenagophila* Taim is totally resistant to *S. mansoni* infection, LE or SJ strain (Santos et al. 1979, Martins-Souza et al. 2003), while Cabo Frio strain is highly susceptible to *S. mansoni* SJ and is partially resistant to *S. mansoni* LE (Corrêa et al. 1979, Martins-Souza et al. 2003).

Infection of snails - Hamsters (*Mesocricetus auratus*) infected with the LE strain of *S. mansoni* were sacrificed 45-50 days after infection, for obtention of miracidia. The snails were individually infected with 20 miracidia, accord-

ing to the technique described by Pellegrino and Katz (1968). Snails with 12-14 mm diameter in size were used in all experiments, at the moment of infection.

Labelling of molecules expressed on the surface of circulating hemocytes - Labelling on the surface of circulating hemocytes from different strains of *Biomphalaria* was performed with lectins isolated from Concanavalin A - ConA, *Glycine max* - SBA, *Triticum vulgare* - WGA and *Arachis hypogaea* - PNA conjugated with fluorescein isothiocyanate - FITC (EY Laboratories, San Mateo). The cells presenting fluorescence after treatment were considered as labelled.

Circulating hemocytes were isolated from the total hemolymph collected, by means of cardiac puncture, from *B. glabrata* or *B. tenagophila* (Cabo Frio and Taim), uninfected, and after 5, 24, 72, and 120 h post-infection with 20 miracidia of *S. mansoni*, according to the technique described by Martins-Souza et al. (2003).

At each experimental point, as well as for each snail strain, analyses were performed with hemocytes, collected and gathered together, from the total hemolymph of 15 snails. Hemocytes present in each group were separated from the hemolymph by centrifugation ($200 \times g$ for 5 min, at room temperature), washed with Chernin's balanced salt solution - CBSS (Chernin 1970), glycerol and trehalose-free (incomplete CBSS). After that, the sediment of hemocytes was resuspended into 1 ml incomplete CBSS, containing 2% bovine albumin. A sample of each group was diluted into 1/10 CBSS containing 0.4% Trypan Blue. Total counting and cellular viability of each sample were estimated in Neubauer's chamber. It was observed that the hemocytes used showed more than 90% viability. Throughout countings, the hemocytes were morphologically classified as small, medium, and large.

Each treatment was carried out in triplicate using 1×10^5 hemocytes for each replica, the hemocytes being distributed into 1.5-ml polypropylene tubes. Simultaneously, a control was performed with 1×10^5 hemocytes, in triplicate, for each labelling in relation to the lectin-hemocyte specificity. After distribution of hemocytes, the tubes were centrifuged ($200 \times g$ for 3 min), the cells resuspended in 200 μ l incomplete CBSS containing 2% bovine albumin and 10 μ g/ml lectin conjugated with FITC (Con A-FITC, WGA-FITC, SBA-FITC or PNA-FITC), and incubated at room temperature, in the dark, for 1 h. The labelled hemocytes in the control tubes were centrifuged and resuspended in incomplete CBSS containing 0.2M of specific carbohydrates for each lectin. Mannose was the carbohydrate used for treatments with Con A-FITC, whereas N-acetyl-glycosamine was utilized for treatments with WGA-FITC and D-galactose in the competition trials with the lectins PNA-FITC and SBA-FITC.

After incubation with labelled lectins and addition of carbohydrates related to the control treatments, the hemocytes were washed and resuspended in 100 μ l incomplete CBSS for analysis under epifluorescence microscope using the appropriate filters (Olympus IX70). A representative number of digital images of hemocytes were obtained from each treatment by means of a digital camera Optronics model DEI-470 and the processing image

software Image Pro-Plus 5.0. For each treatment, digital images were used to analyze at least 100 hemocytes, defining cell type (small, medium or large) and presence of label.

Statistical analysis - The results obtained were statistically analyzed, using analysis of variance or Student's t test for the parametric data, as well as Krushal-Wallis test for non-parametric data.

RESULTS

Circulating hemocytes isolated from *B. glabrata* (BH strain) and *B. tenagophila* (Cabo Frio and Taim strain) expressed ligands that were able to bind to tested lectins Con A, WGA, SBA, and PNA. However, the proportion of labelled hemocytes in relation to each tested lectin was statistically different concerning the species and strains of *Biomphalaria* (Fig. 1). Specifically, about 50% hemocytes from uninfected *B. glabrata* and *B. tenagophila* Cabo Frio were labelled by ConA, however a statistically lower rate (30%) of circulating hemocytes from *B. tenagophila* Taim presented carbohydrates recognized by ConA. In contrast, the proportion of circulating hemocytes labelled with PNA-FITC was statistically higher in hemocytes isolated from *B. tenagophila* Taim (64% of the total) compared with the result obtained in hemocytes from *B. tenagophila* Cabo Frio and *B. glabrata* (24 and 10% of total circulating hemocytes, respectively), and similar pattern was also observed in circulating hemocytes labeled by SBA. Finally, the proportion of circulating hemocytes labelled by WGA-FITC was high, mainly in cells isolated from *B. tenagophila* (70-75% of cells).

Specificity of lectin binding to hemocytes was confirmed by addition of concentrated carbohydrate solution: Mannose for Con A, N-acetylglucosamine for WGA, and Galactosamine for PNA or SBA. In all cases, treatment with the corresponding carbohydrate presented as a result elimination or significant reduction of the fluoresce labelling of hemocytes.

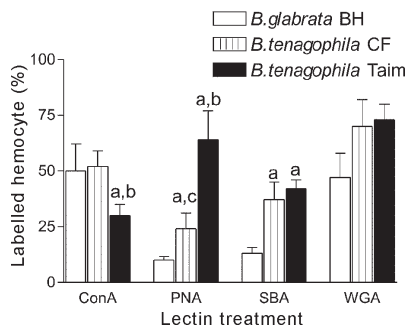


Fig. 1: percentage of total circulating hemocytes isolated from non-infected *Biomphalaria glabrata* BH strain, *B. tenagophila* Cabo Frio strain, and *B. tenagophila* Taim strain, labeled by FITC conjugated ConA, PNA, SBA or WGA purified lectins. a: represents a value significantly different from the value obtained with hemocytes from *B. glabrata* BH; b: represents a value significantly different ($p < 0.05$) from the value obtained with hemocytes from *B. tenagophila* Cabo Frio, and c: represents a value significantly different from the value obtained with hemocytes from *B. tenagophila* Taim.

Besides the difference in the proportion of hemocytes labelled by different lectins, it was possible to observe that there were differences in the labelling pattern and in the morphological type of labelled hemocytes between the different strains or species of tested *Biomphalaria*. In hemocytes isolated from uninfected *B. glabrata*, a diffuse and weak labelling of the cells could be observed, being lectins WGA-FITC (Fig. 2a, b) and ConA-FITC (Fig. 2c, d) bound to carbohydrates on the surface of small and medium hemocytes, whereas only few small cells were labelled by PNA-FITC (Fig. 2e, f). In contrast, hemocytes isolated from uninfected *B. tenagophila* (Taim and Cabo Frio) showed a more intense label than in *B. glabrata*, with large cells presenting a strong labelling on the membrane by WGA (Figs 3b, 4b), while small circulating hemocytes were diffused labeled by ConA (Fig. 3d). Another marked difference was the high number of large and medium hemocytes labelled by PNA-FITC, especially in *B. tenagophila* Taim (Fig. 4c).

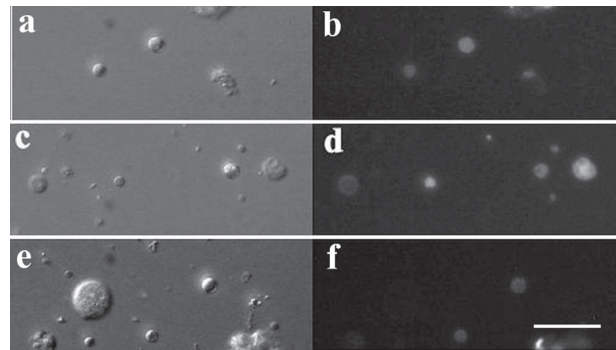


Fig. 2: labelling pattern of circulating hemocytes isolated from non-infected *Biomphalaria glabrata* BH strain, after incubation with FITC conjugated purified lectins. a and b: hemocytes labeled with WGA-FITC; c and d: hemocytes labeled with PNA-FITC; e and f: hemocytes labeled with ConA-FITC; a, c, and e show the cells on Nomarski microscopy while b, d, and f represents the same field on fluorescence microscopy (Bar = 10 μ m).

Attempting to verify variations on the surface of circulating hemocytes that could alter the binding of lectins during *S. mansoni* infection, hemocytes were isolated from different species and strains of *Biomphalaria*, after the first 120 h post-infection, and incubated with lectin conjugated with FITC. *S. mansoni* infection induced an increase in the proportion of hemocytes from *B. glabrata* labelled by Con A. The increase occurred between 5 and 24 hpi, as a consequence of the higher number of small and medium labelled hemocytes. After 72 hpi, the proportion of hemocytes labelled by ConA decreased to a similar level of that observed in uninfected snails. However, only small hemocytes showed labelling, while medium and large ones labelled by lectin were nearly not observed. Reduction in the proportion of circulating hemocytes from *B. glabrata* labelled by Con A was more intense after 120 hpi (Fig. 5a). *S. mansoni* infection also induced a little increase in the proportion of *B. glabrata* hemocytes la-

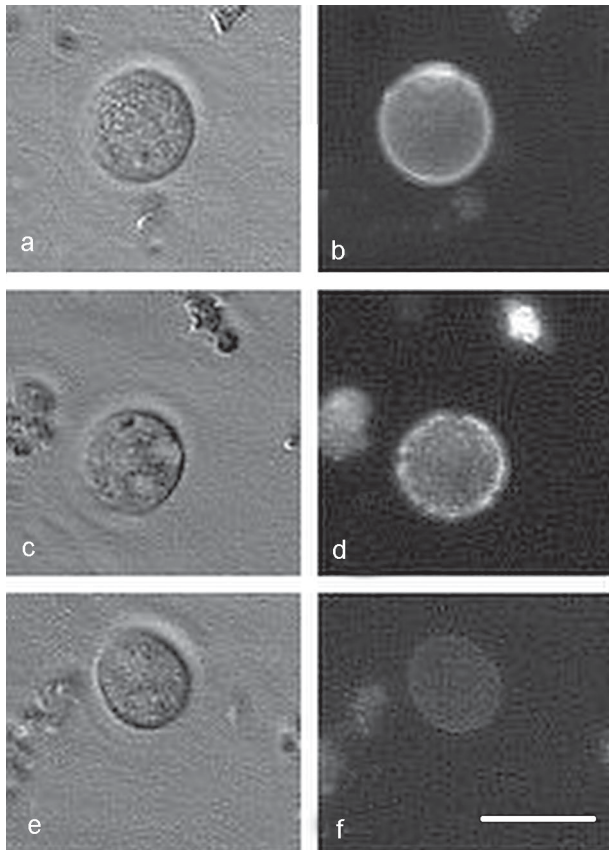


Fig. 3: labelling pattern of circulating hemocytes isolated from non-infected *Biomphalaria tenagophila* Cabo Frio strain, after incubation with FITC conjugated purified lectins. a and b: hemocytes labeled with WGA-FITC; c and d: hemocytes labeled with PNA-FITC; e and f: hemocytes labeled with ConA-FITC; a, c, and e show the cells on Nomarski microscopy while b, d, and f represents the same field on fluorescence microscopy (Bar = 10 µm).

belled by PNA-FITC and SBA-FITC, being this labelling almost exclusively due to small hemocytes, and that increase was maintained high throughout the observation period (Fig. 5b, c). Analyzing the labelling pattern of *B. glabrata* hemocytes by WGA-FITC, it was observed a high number of labelled cells, being most of them represented by small hemocytes. Nevertheless, the number of medium and large hemocytes that were labelled by WGA was considerably higher than the number of hemocytes labelled by other lectins. During infection, it was detected a decrease in the number of medium hemocytes labelled after 5 h post-infection, as well as an increase in the number of large hemocytes labelled after 72 h (Fig. 5d).

S. mansoni infection did not increase the proportion of circulating hemocytes isolated from *B. tenagophila* (Cabo Frio strain) and labelled by ConA-FITC (Fig. 6a) or by WGA-FITC (Fig. 6d). However, it was observed that there was an increase in the proportion of hemocytes labelled by PNA-FITC and SBA-FITC, as a result of the increase in small hemocytes that expressed ligands to these lectins (Fig. 6b, c). Moreover, it is interesting to note that large hemocytes labelled by PNA and WGA nearly disap-

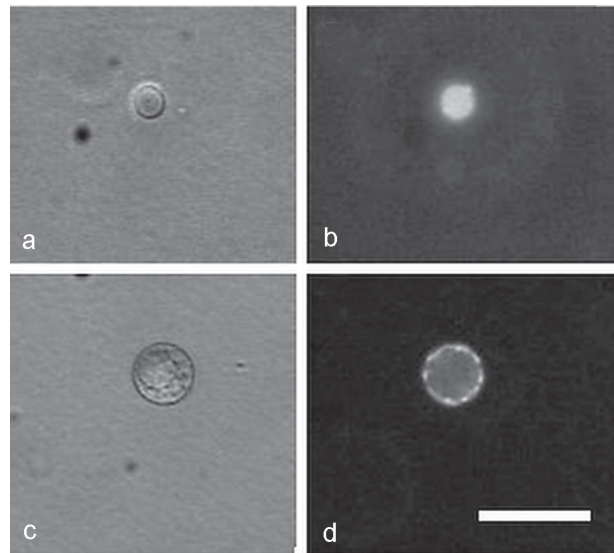


Fig. 4: labelling pattern of circulating hemocytes isolated from non-infected *Biomphalaria tenagophila* Taim strain, after incubation with FITC conjugated purified lectins. a and b: hemocytes labeled with WGA-FITC; c and d: hemocytes labeled with PNA-FITC; a and c show the cells on Nomarski microscopy while panels b and d represents the same field on fluorescence microscopy (Bar = 10 µm).

peared from circulation after 5 h post-infection. Conversely, the proportion of circulating hemocytes labelled by ConA-FITC, PNA-FITC, SBA-FITC, and WGA-FITC decreased after 5 h post-infection with *S. mansoni* in *B. tenagophila* Taim (Fig. 7). Only in labelling by ConA it could be observed an increase in the proportion of hemocytes labelled after 24 and 72 hpi, in relation to non-infected snails (Fig. 7a).

DISCUSSION

Various authors demonstrated that circulating hemocytes in hemolymph of *Biomphalaria* is a heterogeneous cell population. Heterogeneity of these cells was confirmed morphologically (Sminia 1981, Joky et al. 1983, Barraco et al. 1993, Bezerra et al. 1997, Matricón-Gondran & Letorcart 1999, Johnston & Yoshino 2001), and functionally (Granath & Yoshino 1983, Matricón-Gondran & Letorcart 1999b, Martins-Souza et al. 2003). Our results confirmed that circulating hemocytes express ligands (carbohydrates) for lectin binding in a very heterogeneous manner. It is interesting to note that the results demonstrated a great variety detected in the pattern and in the type of binding of lectins in hemocytes from *B. glabrata* when compared to *B. tenagophila*. A high proportion of hemocytes isolated from *B. tenagophila* showed a marked labelling by different types of lectin, specially PNA and WGA, whereas in circulating hemocytes of *B. glabrata* only incubation with ConA presented as a result a high proportion of labelled cells. Some differences were also detected in the labelling pattern of circulating hemocytes isolated from Cabo Frio and Taim strains. Binding between lectins and the surface of hemocytes showed to be specific, since labelling

was inhibited by a high concentration of the corresponding carbohydrate.

Histological studies carried out with different strains of infected *B. glabrata* or *B. tenagophila* has documented that in highly susceptible molluscs there are sporocysts and cercariae in abundance in the host's tissue, and an

early absolute lack of cellular reaction around the parasite. At the other extreme, there are resistant snails, shedding fewer cercariae, but showing an intense hemocyte infiltration in the host's tissue, frequently found around parasitary structures under disintegration (Guaraldo et al. 1981, Sullivan & Richards 1981, Souza et al. 1995, 1997).

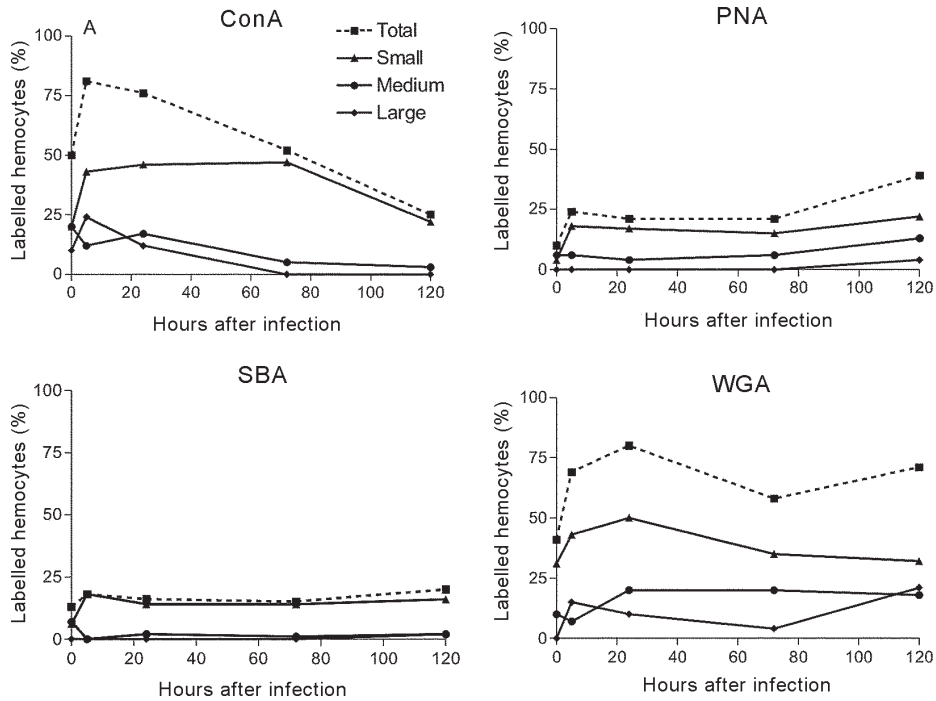


Fig. 5: kinetics of labelled hemocytes of *Biomphalaria glabrata* BH strain, after incubation with FITC conjugated purified lectins, during the first 120 h of *Schistosoma mansoni* infection.

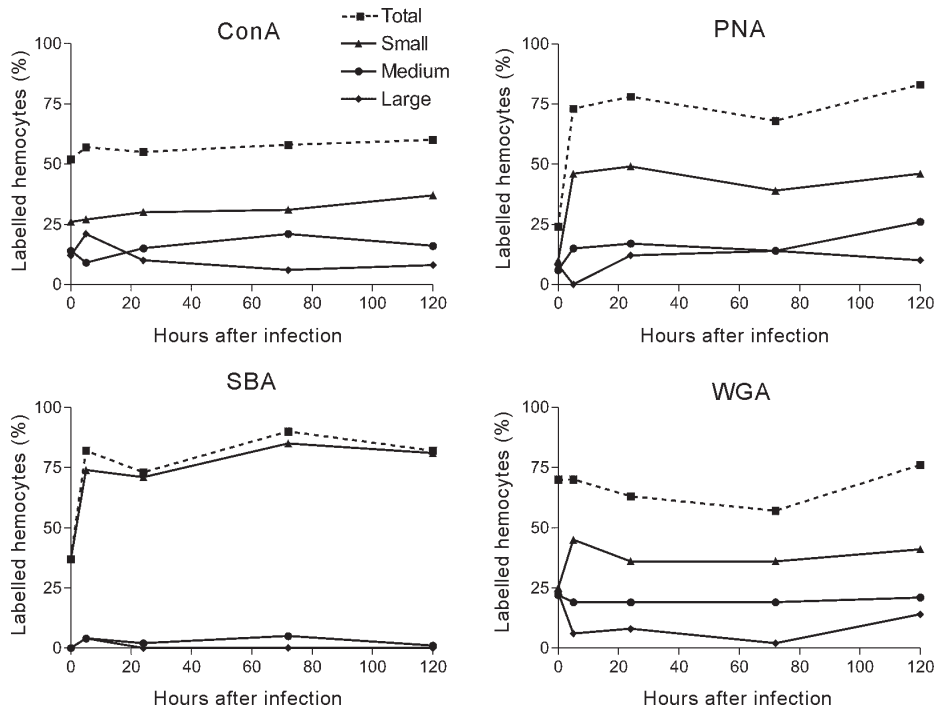


Fig. 6: kinetics of labelled hemocytes of *Biomphalaria tenagophila* Cabo Frio strain, after incubation with FITC conjugated purified lectins, during the first 120 h of *Schistosoma mansoni* infection.

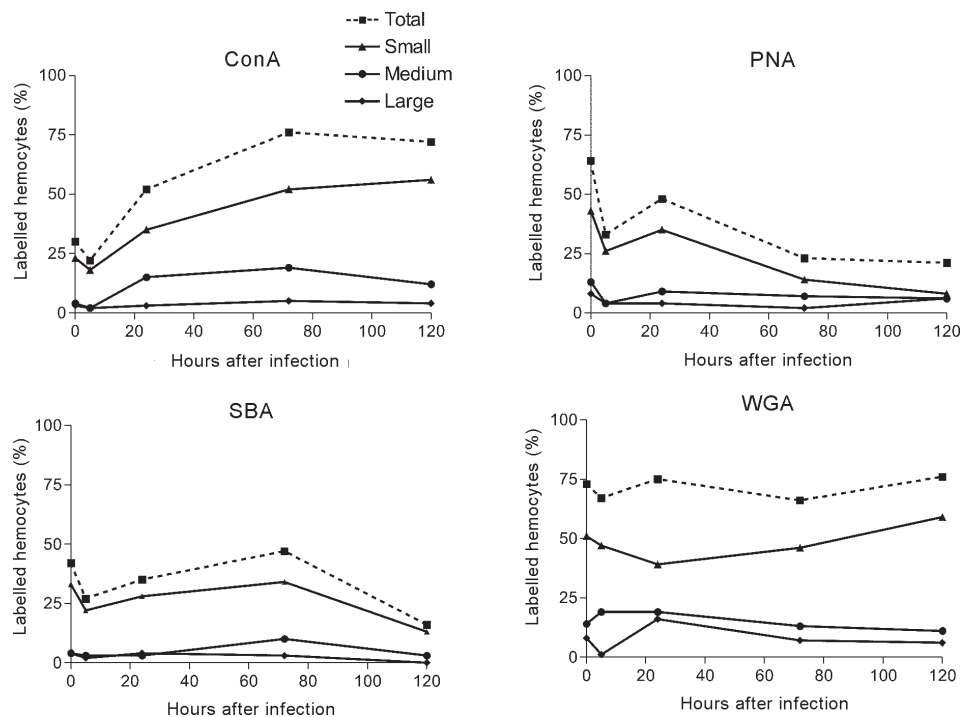


Fig. 7: kinetics of labelled hemocytes of *Biomphalaria tenagophila* Taim strain, after incubation with FITC conjugated purified lectins, during the first 120 h of *Schistosoma mansoni* infection.

Therefore, the significant variation on labelling pattern by lectin observed among different species and strains of *Biomphalaria* used in this study could help to explain hemocyte binding around the parasite observed in some snail strain and, therefore it would be an important factor for determination of differences in the susceptibility level to *S. mansoni* infection reported in the snail species (Corrêa et al. 1979, Martins-Souza et al. 2003, Coelho et al. 2004, Pereira 2005, Rosa et al. 2005).

Lectin/carbohydrate binding has been associated with recognition of trematode larvae, and could exert influence upon the hemocyte capacity of encapsulating trematode larvae (van der Knaap & Lokert 1990, Yoshino et al. 2001, Guillou et al. 2004). In this sense, various authors demonstrated that the tegument, at the larval stages of trematodes, is coated with a high level of glycosidic compounds, and the majority of the tested lectins were specifically able to bind either to axenically transformed larvae (Uchikawa & Loker 1991) or to proteins isolated from its tegument (Johnston & Yoshino 1996). On the other hand, the presence of lectins has been detected in hemolymph of *Biomphalaria*. However, it was not possible to associate the presence of lectins in snail hemolymph with the susceptibility level to *S. mansoni* infection of the snail strain (Zelck et al. 1995, Johnston & Yoshino 1996). Our results show that there were differences in the expression of haptens (glycoproteins or glycolipids) on the hemocyte membrane of different species or strains of *Biomphalaria*. These differences could result in greater capacity of hemocytes, from different strains, of recognizing and adhering to the parasite.

Corroborating this hypothesis, our results also demonstrate that *S. mansoni* infection produced dissimilar effects on the labelling pattern of circulating hemocytes by lectins in different snail strains. In *B. glabrata*, it was observed that there was a significant increase on the proportion of circulating hemocytes labelled by lectins, up to 72 h post-infection. Conversely, 5 h after *S. mansoni* infection, the hemocytes labelled by lectins nearly disappeared from the circulation of *B. tenagophila* Taim, a strain able to destroy sporocysts completely. Previous works (Bezerra et al. 1997, Martins-Souza et al. 2003) report a decrease in the total number of circulating hemocytes in *B. glabrata* or *B. tenagophila* at the first hours after infection. However, the results here presented indicate that hemocytes which are out of circulation in *B. tenagophila* Taim are the ones that express haptens for lectins, suggesting that these hemocytes could be retained in the site of infection, thus justifying the intense cellular reaction observed in these snails that would result in early parasite destruction.

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