Participation of N-acetyl-D-glucosamine carbohydrate moieties in the recognition of *Schistosoma mansoni* sporocysts by haemocytes of *Biomphalaria tenagophila*

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Lectin-carbohydrate binding may be involved in the recognition of Schistosoma mansoni *sporocysts by haemocytes of* Biomphalaria*; therefore, we tested if this interaction is associated with snail resistance against* Schistosoma *infection. In vitro data showed that most of the* S. mansoni *sporocysts cultured with haemocytes from* Biomphalaria glabrata *BH, a highly susceptible snail strain, had a low number of cells that adhered to their tegument and a low mortality rate. Moreover, the addition of N-acetyl-D-glucosamine (GlcNAc) did not alter this pattern of adherence and mortality. Using haemocytes and haemolymph of* Biomphalaria tenagophila *Cabo Frio, we observed a high percentage of sporocysts with adherent cells, but complete encapsulation was not detected. Low concentrations of GlcNAc increased haemocyte binding to the sporocysts and mortality, which returned to basal levels with high concentrations of the carbohydrate. In contrast, haemocytes plus haemolymph from* B. tenagophila *Taim encapsulated cellular adhesion index of level 3 and destroyed over 30% of the* S. mansoni *sporocysts in culture. Interestingly, the addition of GlcNAc, but not mannose, to the culture medium resulted in the significant inhibition of cellular adhesion to the parasite tegument and the reduction of parasite mortality, suggesting that GlcNAc carbohydrate moieties are important to the recognition of* S. mansoni *by* B. tenagophila *Taim.*

Key words: *Schistosoma mansoni* - *Biomphalaria tenagophila* - snail susceptibility to trematode - N-acetyl-D-glucosamine - host-parasite recognition

Schistosomiasis affects more than 200 million people worldwide, most frequently in populations of developing countries in Africa, Asia and Latin America (Gryseels et al. 2006). In Brazil, it is estimated that 6-8 million people are infected by *Schistosoma mansoni* (Katz & Peixoto 2000, WHO 2002). The transmission of schistosomiasis in human populations has been associated with environmental and socio-economic conditions, but the presence of susceptible snail strains in the area is obligatory (Pereira et al. 2010). Experimental studies have indicated that compatibility between the snail and Digenea is determined, in part, by the degree of the snail defence response against the parasite infection (Souza et al. 1997, Negrão-Corrêa et al. 2007, Bayne 2009, Hanington et al. 2010b). Therefore, a better understanding of the schistosome-snail interaction is essential for the development of new strategies for schistosomiasis transmission control (Coelho et al. 2004).

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In *Biomphalaria* species, the internal defence system responses to trematode larvae are partially dependent on the capability of the haemocytes to recognise sporocyst tegument molecules, leading to cellular activation and the production of highly toxic metabolites of oxygen and nitrogen that are associated with parasite mortality (Hahn et al. 2000, 2001, Bender et al. 2005, Bayne 2009, Moné et al. 2010). In this context, the first step in the activation of this defence mechanism is the recognition of the parasite by haemocytes. The tegument of *S. mansoni* sporocysts is composed of highly glycosylated (Yoshino 1977, Uchikawa & Locker 1991, Johnston & Yoshino 1996) molecules that bind to soluble proteins of the *Biomphalaria glabrata* haemolymph in a carbohydrate-dependent manner (Johnston & Yoshino 1996). Furthermore, it has been demonstrated that excretory-secretory glycoproteins from *S. mansoni* sporocysts also bind to haemocytes via carbohydrate-binding receptors (Johnston & Yoshino 2001). Therefore, lectin-carbohydrate binding could functionally mediate the association of haemocytes with the trematode tegument (Van der Knaap & Locker 1990, Johnston & Yoshino 2001, Bayne 2009); consequently, this binding could be a determining factor of *Biomphalaria* susceptibility to *S. mansoni* infection. The better-known lectins in the haemolymph of *B. glabrata* are members of a family of somatically diversified fibrinogen-related proteins (FREPs) (Adema et al. 1997, Zhang et al. 2008). FREPs are calcium-dependent lectins that contain one

or two N-terminal immunoglobulin-like domains and a C-terminal fibrinogen domain. These lectins are up-regulated after trematode infection and associated with glycan-bearing molecules released by the parasite larvae (Zhang et al. 2008, Hanington et al. 2010a, b, Moné et al. 2010). Recent work has demonstrated that *B. glabrata* FREP3 has opsonic properties against haemocytes and that knocking down FREP3 resulted in an alteration of the snail resistance to *Echinostoma paraensei* infection (Hanington et al. 2010b). However, FREPs have not been identified in other *Biomphalaria* species and their function in parasite recognition and destruction has not yet been well defined (reviewed by Bayne 2009). Although lectin-carbohydrate binding has been clearly associated with sporocyst recognition and haemocyte activation by *B. glabrata* (Hahn et al. 2000, Castillo & Yoshino 2002), there has been no experimental evidence showing the participation of carbohydrates in *S. mansoni* recognition by haemocytes from other *Biomphalaria* species involved in schistosomiasis transmission, such as *Biomphalaria tenagophila*.

Previous work has demonstrated that a *B. tenagophila* strain isolated from the Biological Reservoir of Taim, designated as the Taim strain, is completely resistant to *S. mansoni* infection (Corrêa et al. 1979, Santos et al. 1979). *S. mansoni* penetration in this snail strain induced an intense cellular infiltration at the infection site leading to parasite destruction during the first 24 h of the infection (Negrão-Corrêa et al. 2007). Moreover, most of the circulating haemocytes recovered from *B. tenagophila* Taim were intensely labelled by fluorescein isothiocyanate (FITC)-conjugated PNA and WGA lectins and these labelled cells almost disappeared from the circulation after *S. mansoni* infection (Martins-Souza et al. 2006). Therefore, in this experimental work, we tested the role of a competing carbohydrate, N-acetyl-D-glucosamine (GlcNAc), in the in vitro haemocyte-parasite interaction that leads to the adhesion and destruction of *S. mansoni* sporocysts by circulating haemocytes isolated from *B. glabrata* and *B. tenagophila*.

MATERIALS AND METHODS

Parasites - The LE strain of *S. mansoni*, originally isolated from a patient in Belo Horizonte, state of Minas Gerais, Brazil, was used in all of the experiments. This parasite strain has been maintained in the Laboratory of Schistosomiasis, Department of Parasitology, Federal University of Minas Gerais (ICB/UFMG) by successive passages in *B. glabrata* and hamsters (*Mesocricetus auratus)* since its isolation in 1968 (Pellegrino & Katz 1968).

Snails - The strains of *B. glabrata* or *B. tenagophila* used in this study have been bred and maintained according to the procedures previously described by Pellegrino and Katz (1968) at the Laboratory of Schistosomiasis (ICB/UFMG). The BH strain of *B. glabrata* used in our experiments was isolated from Pampulha Lake, Belo Horizonte, and it is highly susceptible to *S. mansoni* infection (Paraense & Corrêa 1963). Two strains of *B. tenagophila* were selected for this study. The Taim strain collected at the Ecological Station of Taim, state of Rio Grande do Sul, Brazil and the Cabo Frio strain collected in Cabo Frio, state of Rio de Janeiro, Brazil. *B. tenagophila* Taim is completely resistant to *S. mansoni* infection (Corrêa et al. 1979, Santos et al. 1979, Martins-Souza et al. 2003, Coelho et al. 2004, Rosa et al. 2005), whereas the Cabo Frio strain is partially susceptible to *S. mansoni* LE (Corrêa et al. 1979, Martins-Souza et al. 2003).

Axenic transformation of S. mansoni sporocysts - *S. mansoni* miracidia were obtained from the liver of infected hamsters under axenic conditions and isolated using the procedures described by Chaia (1956). Newly hatched miracidia were washed and concentrated on ice in 15-mL conical polypropylene tubes containing cooled Chernin's balanced salt solution [Chernin's balanced salt solution (CBSS) buffer; 48 mM NaCl, 2.0 mM KCl, 0.5 mM Na_2HPO_4, 1.8 mM $MgSO_4.7H_2O$, 3.6 mM $CaCl_2.2H_2O$, 0.6 mM $NaHCO_3$, 5.5 mM glucose and 3 mM trehalose, pH 7.4]. Miracidia were then transferred to 50 mL-culture flasks containing Roswell Park Memorial Institute-1640 medium (Sigma-Aldrich) supplemented with 5% foetal bovine serum and was incubated at 27ºC with 5% CO_2 for 24 h for sporocyst transformation (Samuelson et al. 1984). Transformed sporocysts were washed in CBSS buffer and re-suspended in supplemented CBSS medium containing 2% bovine serum albumin (BSA), 25 mM HEPES, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma, St. Louis MO, USA) and 1% minimum essential medium amino acid solution (Atlanta Biologicals, Norcross GA, USA) for use in subsequent in vitro assays.

Haemolymph collection and haemocyte separation - Total haemolymph was collected by cardiac puncture (Zelck et al. 1995) from a pool of 15-20 non-infected *B. glabrata* or *B. tenagophila* (Cabo Frio and Taim strains) measuring 10-14 mm in diameter, as detailed by Martins-Souza et al. (2003). Haemocytes were separated from the haemolymph by centrifugation (200 *g* for 5 min at room temperature), washed with CBSS buffer and the cell sediment was resuspended in 1 mL CBSS buffer. A sample of the cell suspension was diluted 1:9 in CBSS containing 0.4% trypan blue and the total counting and cellular viability of each sample were estimated in Neubauer's chambers. The haemocyte suspensions used in each experimental procedure showed more than 90% viability. The soluble fraction of haemolymph, called acellular haemolymph, was further centrifuged at 3,000 *g* for 30 min at 4ºC and maintained on ice for less than 30 min until use in the in vitro assays.

Interaction of Biomphalaria haemocytes with S. mansoni sporocysts - Haemocytes of each snail strain and *S. mansoni* sporocysts were combined in 96-well culture plates at a density of 1 x 10^5 cells and 50 sporocysts in CBSS complete medium to a final volume of 200 µL. The assay was also performed in the presence of complete CBSS containing 10% acellular haemolymph collected from the same snail group as that of the haemocytes. To evaluate the role of GlcNAc in haemocyte binding to sporocyst teguments, we performed the haemocyte-sporocyst interaction assay in the presence of increasing concentrations of GlcNAc (0, 1, 5, 25 and 100 mM). To verify the specific effect of GlcNAc on haemocyte ad-

hesion to sporocysts, the haemocyte-sporocyst interaction assay was also performed in the presence of 50 mM mannose. As a negative control, *S. mansoni* sporocysts were incubated with supplemented CBSS alone. Each treatment and control was performed in triplicate and independently replicated at least twice. For each treatment, there was one cell mortality control containing haemocytes and culture medium with increasing concentrations of GlcNAc. The plates were incubated at 26ºC for 6 h. At this time, the plates were examined under an inverted microscope (Olympus IX70) to estimate the adhesion of haemocytes to living *S. mansoni* sporocysts and the number of dead and dying sporocysts. The mortality rate of haemocytes was also evaluated using trypan blue staining as described above.

Cell adhesion index (CAI) - The CAI was used to evaluate the level of haemocyte binding to axenically transformed *S. mansoni* sporocysts based on the protocol of Castillo and Yoshino (2002). Briefly, a CAI = 0 was assigned to the sporocysts that had no cells bound on their surface (Fig. 1A), CAI = 1 for sporocysts that had 1-10 cells attached to their surface (Fig. 1B), CAI = 2 for sporocysts that had 11-50 cells attached to their surface (Fig. 1C) and CAI = 3 for sporocysts that had more than 50 haemocytes attached to their surface (Fig. 1D).

CAI values were estimated under an Olympus IX70 in sporocysts cultured for 6 h. Evaluation was performed on the digital images of sporocysts obtained from each treatment using a digital camera (Optronics model DEI-470) and the processing image software Image Pro-Plus 5.0. At least 10 fields were photographed for each treatment.

Sporocyst viability - After 6 h of incubation, the sporocyst mortality rate was assessed by the incorporation of trypan blue stain (0.4% trypan blue in CBSS), as detailed by Pereira et al. (2008).

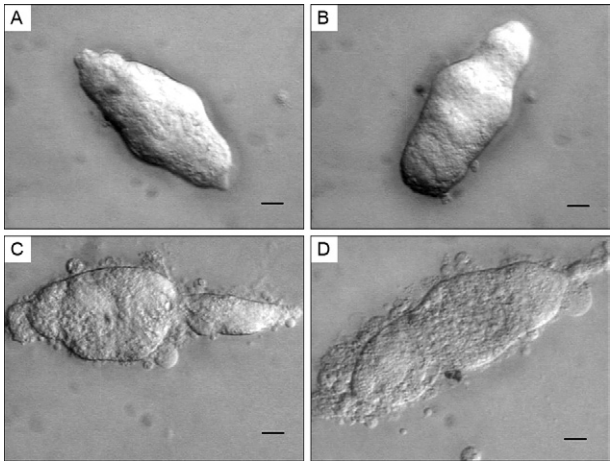

Fig. 1: the cell adhesion index (CAI). A: photo microscopy representative of the CAI = 0 score, with no haemocytes adhered to the sporocyst tegument; B: CAI = 1, with less than 10 haemocytes adhered to the sporocyst tegument; C: CAI = 2, between 10-50 haemocytes adhered to the sporocyst tegument; D: CAI = 3, with more than 50 haemocytes adhered to the sporocyst tegument, characterizing encapsulation. Bar = 10 μm.

Statistical analysis - Normally distributed data are reported as the means ± standard deviation and analysed by Student's *t* test or one-way analysis of variance. In the latter analysis, p values were assigned using the Tukey test. Differences of $p < 0.05$ were considered significant. For the data with distributions other than normal, the non-parametric method of Kruskal-Wallis was used.

RESULTS

The interaction of haemocytes from different *Biomphalaria* species or strains with *S. mansoni* sporocysts were comparatively evaluated in vitro by quantifying the haemocyte adhesion to the parasite larvae and the parasite mortality rate after 6 h in culture. Most of the *S. mansoni* sporocysts cultured with circulating haemocytes obtained from the non-infected *B. glabrata* BH strain showed no haemocyte adhesion to the tegument of the larvae (Fig. 2A). Specifically, 67% of the sporocysts cultured in the presence of *B. glabrata* haemocytes had a CAI = 0 and the remaining sporocysts (33%) had a small number of haemocytes bound to their tegument (CAI = 1). The adhesion of haemocytes to sporocysts did not change in the presence of acellular haemolymph from the same snail strain (Fig. 2B). In addition to the low haemocyte adhesion, sporocysts cultured with *B. glabrata* BH haemocytes also had low mortality rates, reaching only 5% of the *S. mansoni* sporocysts after 6 h of culture with haemocytes or haemocytes plus haemolymph (Fig. 2C).

In contrast, most of the *S. mansoni* sporocysts cultured with circulating haemocytes isolated from *B. tenagophila* showed cellular adhesion to the parasite tegument and this adhesion increased when acellular haemolymph from the same snail strain was added to the culture medium (Fig. 2A, B). In the presence of haemocytes from *B. tenagophila* Cabo Frio, we observed that 50% of the sporocysts showed haemocytes adhering to the parasite tegument and this value increased to 90% after the addition of acellular haemolymph from same snail strain to the culture medium. However, no sporocysts with a CAI = 3, which indicates parasite encapsulation, were observed under this treatment condition (Fig. 2A, B). In contrast, a very high level of haemocyte adhesion to the parasite tegument (> 90% of the sporocysts) was detected in culture containing haemocytes isolated from *B. tenagophila* Taim, the resistant parasite strain (Fig. 2A, B). Interestingly, all *S. mansoni* sporocysts cultured in the presence of haemocytes plus haemolymph from *B. tenagophila* Taim had haemocytes that adhered to their tegument and more than 30% of the sporocysts had a CAI = 3, indicative of a complete encapsulation of the parasite by these haemocytes (Fig. 2B). Sporocyst mortality in culture medium containing haemocytes from *B. tenagophila* Cabo Frio was 8% ± 1.4 and significantly increased to 15% ± 1 with the addition of acellular haemolymph. Sporocyst cultures containing haemocytes from *B. tenagophila* Taim had a high mortality rate (13%), which significantly increased to 28% after the addition of acellular haemolymph (Fig. 2C).

We have previously shown that haemocytes from *B. tenagophila*, but not from *B. glabrata*, are intensely labelled with WGA lectin conjugated to FITC (Martins-

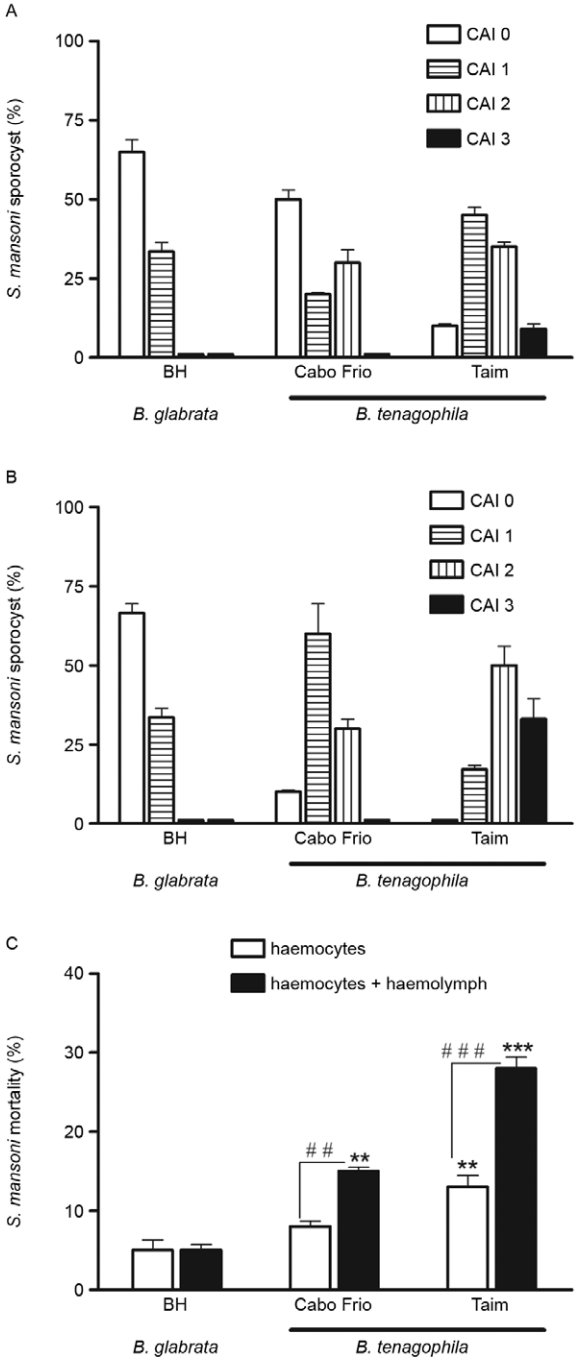

Fig. 2: haemocytes adhesion and mortality rate of *Schistosoma mansoni* primary sporocysts cultured with circulating haemocytes from non-infected *Biomphalaria glabrata* BH strain, from *Biomphalaria tenagophila* Cabo Frio strain or from *B. tenagophila* Taim strain. Circulating haemocytes (1 x 10^5) and 50 *S. mansoni* sporocysts axenically transformed were plated into 96-well culture plates containing Chernin's balanced salt solution complete medium with or without 10% of acellular haemolymph of the same snail. The cellular adhesion to sporocysts tegument in absence of haemolymph (A) or in presence of haemolymph (B) and the parasite mortality (C) was evaluated 6 h after incubation. The values represent the average ± standard deviation of four replicates in each group and it is representative of three independent experiments. In C, ***$p < 0.001$ and **$p < 0.01$ when comparing with the parasite mortality observed in culture containing haemocytes from *B. glabrata* BH. ###$p < 0.001$ and ##$p < 0.01$ between the groups. CAI: cell adhesion index.

Souza et al. 2006); therefore, we tested the effect of GlcNAc on the in vitro haemocyte-parasite interaction. The addition of increasing doses of the carbohydrate to the sporocyst culture medium containing circulating haemocytes from *B. glabrata* BH did not alter the low cellular adhesion to the parasite (Fig. 3A) even in the presence of acellular haemolymph from the same snail strain (Fig. 3B).

In sporocyst cultures containing haemocytes isolated from *B. tenagophila* Cabo Frio, the addition of 25 and 100 mM GlcNAc to the culture medium without haemolymph resulted in an increase in the percentage of sporocysts with a CAI = 1 and a reduction in the percentage of sporocysts with a CAI = 0, but no changes were detected in the percentage of sporocysts with a CAI = 2 or 3 (Fig. 4A). Significant alterations in the CAI = 0 and 1 values were also detected in sporocyst cultures with haemocytes plus haemolymph from Cabo Frio after the addition of 1, 5, 25 and 100 mM GlcNAc (Fig. 4B). Under these culture conditions, the presence of 1, 5 and 25 mM of GlcNAc also resulted in a small number of sporocysts with a CAI = 3 that was blocked in the presence of 100 mM of GlcNAc (Fig. 4B).

Interestingly, the addition of increasing doses of GlcNAc to sporocyst cultures containing haemocytes from *B. tenagophila* Taim resulted in the significant reduction of haemocytes adhering to the parasite tegument, thereby abolishing the encapsulation of the parasite in a dose-dependent manner (Fig. 5A). Similar results were observed in cultures containing haemocytes plus acellular haemolymph of *B. tenagophila* Taim (Fig. 5B). In contrast, an alteration was not detected in the binding of haemocytes *S. mansoni* sporocysts upon the addition of 50 mM mannose (Fig. 6). To ensure that the effect of carbohydrates on haemocyte adhesion to sporocyst teguments was not due to undesirable effects on haemocytes, we tested haemocyte viability after 6 h of incubation in culture me-

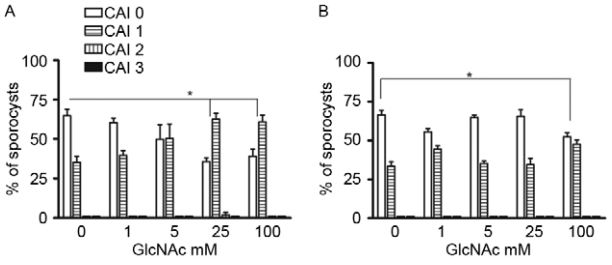

Fig. 3: influence of N-acetyl-D-glucosamine (GlcNAc) in the *Biomphalaria glabrata* haemocyte adhesion to *Schistosoma mansoni* primary sporocysts. Circulating haemocytes (1 x 10^5) from non-infected *B. glabrata* BH strain and 50 *S. mansoni* sporocysts axenically transformed were plated into 96-well culture plates containing Chernin's balanced salt solution complete medium and increasing concentration (1-100 mM) of GlcNAc. The haemocyte adhesion to sporocysts tegument in absence of acellular haemolymph (A) or in presence of acellular haemolymph (B) was evaluated 6 h after incubation. The values represent the average ± standard deviation of four replicates in each group and it is representative of two independent experiments. Asterisk means $p < 0.05$ comparing with the control treatment without GlcNAc. CAI: cell adhesion index.

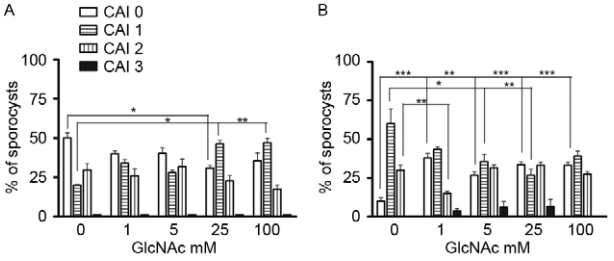

Fig. 4: influence of N-acetyl-D-glucosamine (GlcNAc) in the *Biomphalaria tenagophila* Cabo Frio haemocyte adhesion to *Schistosoma mansoni* primary sporocysts. Circulating haemocytes (1 x 10^5) from non-infected *B. tenagophila* Cabo Frio strain and 50 *S. mansoni* sporocysts axenically transformed were plated into 96-well culture plates containing Chernin's balanced salt solution complete medium and increasing concentration (1-100 mM) of GlcNAc. The haemocyte adhesion to sporocysts tegument in absence of acellular hemolymph (A) or in presence of acellular haemolymph (B) was evaluated 6 h after incubation. The values represent the average ± standard deviation of four replicates in each group and it is representative of two independent experiments. ***p < 0.001, **p < 0.01 and *p < 0.05 comparing with the control treatment without GlcNAc. CAI: cell adhesion index.

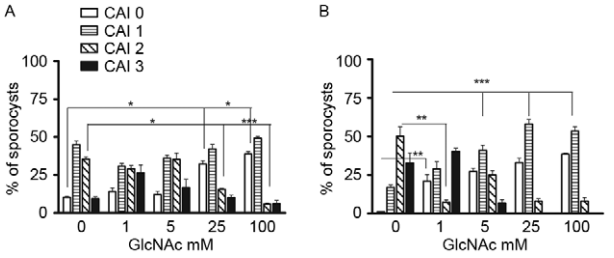

Fig. 5: influence of N-acetyl-D-glucosamine (GlcNAc) in the *Biomphalaria tenagophila* Taim haemocyte adhesion to *Schistosoma mansoni* primary sporocysts. Circulating haemocytes (1 x 10^5) from non-infected *B. tenagophila* Taim strain and 50 *S. mansoni* sporocysts axenically transformed were plated into 96-well culture plates containing Chernin's balanced salt solution complete medium and increasing concentration (1-100 mM) of GlcNAc. The haemocyte adhesion to sporocysts tegument in absence of acellular haemolymph (A) or in presence of acellular haemolymph (B) was evaluated 6 h after incubation. The values represent the average ± standard deviation of four replicates in each group and it is representative of two independent experiments. ***p < 0.001, **p < 0.01 and *p < 0.05 comparing with the control treatment without GlcNAc. CAI: cell adhesion index.

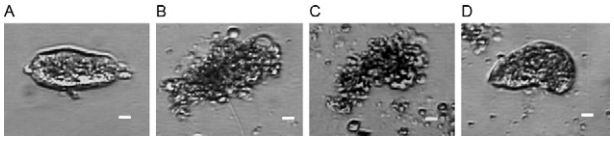

Fig. 6: photomicroscopes representative of *Schistosoma mansoni* sporocysts cultured with circulating haemocytes isolated from *Biomphalaria tenagophila* Taim. A: *S. mansoni* sporocysts in culture medium with no haemocytes added; B: sporocysts of *S. mansoni* cultured in presence of haemocytes from *B. tenagophila* Taim; C: sporocysts of *S. mansoni* and haemocytes from *B. tenagophila* Taim in culture medium containing 50 mM of mannose; D: sporocysts of *S. mansoni* and haemocytes from *B. tenagophila* Taim in culture medium containing 25 mM of N-acetyl-D-glucosamine. Note complete encapsulation of sporocysts in B and C, but few haemocytes bound to parasite tegument in D. Bar = 10 μm.

dium containing increasing concentrations of mannose or GlcNAc. The haemocyte viability rate ranged from 75-80%, independently of the carbohydrate concentration.

We then tested the ability of GlcNAc to affect parasite mortality in culture with haemocytes and acellular haemolymph from different snail strains. As expected, the addition of increasing doses of GlcNAc to sporocysts of *S. mansoni* cultured in the presence of haemocytes from *B. glabrata* BH did not change the low parasite mortality rate detected under these culture conditions (Fig. 7A). In contrast, the addition of GlcNAc to sporocysts in cultures containing haemocytes and haemolymph from *B. tenagophila* affected the parasite mortality rate. The presence of 25 mM GlcNAc significantly increased the parasite mortality rate when cultured with haemocytes from *B. tenagophila* Cabo Frio, but the parasite mortality rate was reduced with high doses of the carbohydrate (Fig. 7B). However, the mortality rate of *S. mansoni* sporocysts incubated with haemocytes and haemolymph from *B. tenagophila* Taim dropped from 28-5% in 25 mM and was 5% in 100 mM of GlcNAc (Fig. 7C).

DISCUSSION

In Brazil, the high prevalence of schistosomiasis in the human population has been associated with the presence of *Biomphalaria* species that are highly susceptible to *S. mansoni*, such as *B. glabrata* (Paraense 2001). Histopathological studies indicated that susceptible snail species or strains have a large number of parasite structures in different tissues without an apparent tissue reaction, whereas in more resistant snails, few (or no) intact sporocysts or cercariae are found in the host tissue and remnants of the parasite are surrounded by a massive accumulation of haemocytes (Newton 1952, Pan 1965, Souza et al. 1995, Negrão-Corrêa et al. 2007). These data suggested that the capability of snail haemocytes to encapsulate the parasite larvae could be associated with resistance to *S. mansoni* infection. If this were the case, we would expect that haemocytes from resistant snails should be more efficient at recognising the mother sporocyst tegument. In agreement with this hypothesis, our data clearly showed that circulating haemocytes isolated from *B. glabrata* BH or *B. tenagophila* Cabo Frio, snail species that are susceptible *S. mansoni* infection, had a low ability to bind to *S. mansoni* sporocysts during the in vitro interaction, in contrast with the high level of parasite encapsulation observed in sporocyst cultures containing haemocytes from *B. tenagophila* Taim, a snail species resistant to with *S. mansoni* infection. Furthermore, the ability of haemocytes to bind and encapsulate parasite larvae was associated with their mortality rate (Fig. 2).

The low level of adhesion of *B. glabrata* haemocytes to the sporocyst tegument detected in our experiments indicated that the parasite is not well recognised by the snail defence system, which would explain the high level of susceptibility to *S. mansoni* observed for this snail strain. Earlier studies (Bayne et al. 1986, Dissous et al. 1986, Damian 1989) suggested that the *S. mansoni* sporocysts cover themselves with host-like antigens to escape from the snail defence system. It is important to note that in our experimental protocol, *S. mansoni* miracidia

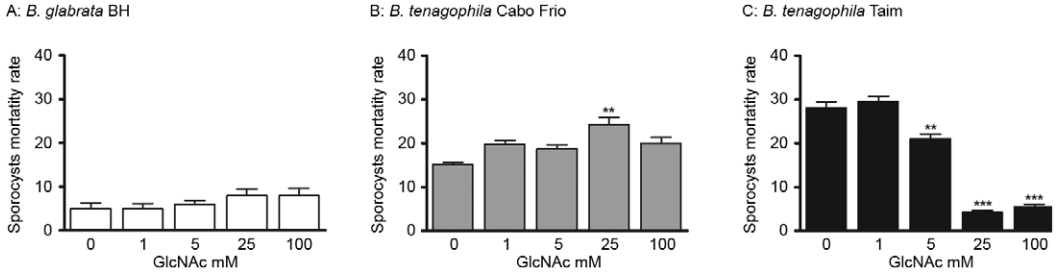

Fig. 7: influence of N-acetyl-D-glucosamine (GlcNAc) in the mortality of *Schistosoma mansoni* primary sporocysts cultured with haemocytes plus acellular haemolymph from *Biomphalaria glabrata* of BH strain (A), or from *Biomphalaria tenagophila* of Cabo Frio strain (B) or from *B. tenagophila* of Taim strain (C). Circulating haemocytes (1 x 10^5) and acellular haemolymph from each snail strain were added to 50 *S. mansoni* sporocysts axenically transformed plated into 96-well culture plates containing Chernin's balanced salt solution complete medium and increasing concentration (1-100 mM) of GlcNAc. The parasite mortality rate was evaluated after 6 h of incubation. The values represent the average ± standard deviation of four replicates in each group and it is representative of two independent experiments. ***$p < 0.001$ and **$p < 0.01$ comparing with the control treatment without GlcNAc.

were axenically transformed and had no previous contact with the intermediate host; therefore, if molecular mimicry does occur in this system, it would be mediated by molecules with high homology produced by the parasite and the intermediate host. More recently, it was demonstrated that glycoproteins from the haemolymph and tissue samples from the snail foot and hepatopancreas of susceptible uninfected *B. glabrata* have carbohydrate structures that cross-react with glycoconjugates from *S. mansoni* miracidia, supporting the concept of carbohydrate-based molecular mimicry as an invasion strategy (Lehr et al. 2008, 2010). Therefore, this strategy would avoid the initial parasite recognition by the haemocyte through carbohydrate binding receptors, leading to establishment of the infections observed in susceptible snail strains.

In contrast, in resistant snail strains, such as *B. tenagophila* Taim, carbohydrate-based molecular mimicry between the parasite and the snail would not be effective, allowing for the recognition of the parasite by circulating haemocytes. In our previous work (Martins-Souza et al. 2006), we showed that circulating haemocytes from non-infected *B. tenagophila* Taim are strongly labelled with FITC-conjugated WGA and PNA lectins and that the labelled cells disappeared from circulation 5 h after *S. mansoni* infection, suggesting a possible participation of the lectin-carbohydrate interaction in the protective mechanism. Therefore, we tested the participation of the lectin-carbohydrate interaction, specifically the WGA ligand, in sporocyst recognition by *B. tenagophila* haemocytes. The results clearly showed that the addition of GlcNAc, but not mannose, blocked haemocyte adhesion to *S. mansoni* sporocysts and reduced the mortality rate of the parasite produced by *B. tenagophila* Taim, the parasite-resistant snail. Moreover, *S. mansoni* sporocyst recognition increased in the presence of cell-free haemolymph, suggesting that soluble carbohydrate-binding molecules would also opsonise the parasite larvae and favour the recognition. In both cases, our data indicated that molecules containing GlcNAc moieties participate in the haemocyte adhesion to the *S. mansoni* tegument and that this interaction was specifically blocked by competition with the free monosaccharide.

The importance of carbohydrate interactions in pathogen recognition by haemocytes of molluscs has been well documented (Van der Knaap & Locker 1990, Castillo et al. 2007, Bayne 2009). Specifically, for *B. glabrata*, Fryer et al. (1989) demonstrated that the phagocytosis of yeast by haemocytes can be inhibited upon incubation with carbohydrates. Subsequently, Johnston and Yoshino (2001) showed that fluorescent-conjugated excretory-secretory glycoproteins (fESP) from *S. mansoni* sporocysts bound to circulating haemocytes of *B. glabrata* (13-16-R1 strain) and that fESP binding to haemocytes was partially inhibited by various glycoconjugates. Furthermore, Castillo and Yoshino (2002) demonstrated that fucoidan and other sulphated glycoconjugates were able to reduce the adhesion index of the *B. glabrata* embryonic cell line (Bge) to *S. mansoni* primary sporocysts. These authors showed that N-glycosidase treatment of the parasite tegument protein abolished the binding to biotinylated Bge cell proteins, suggesting that lectin-carbohydrate interactions could mediate cellular adhesion to the parasite larvae. Recently, Hanington et al. (2010b) demonstrated that FREP3, a secreted lectin that is up-regulated in resistant strains of *B. glabrata* infected by *S. mansoni* or *E. paraensei*, could function as an opsonin to favour haemocyte adhesion. Moreover, siRNA-mediated interference of FREP3 resulted in an increased susceptibility to *E. paraensei*.

Our data demonstrated that lectin-carbohydrate interactions participate in sporocyst recognition by haemocytes from *B. tenagophila*, as has already been shown in *B. glabrata*. In addition, our data suggest that different glycoconjugates could be involved in parasite recognition by *B. tenagophila* and *B. glabrata*. In haemocytes from *B. glabrata*, Hahn et al. (2000) showed that BSA-carbohydrate conjugates, such as BSA-galactose, BSA-mannose and BSA-fucose, but not BSA-GlcNAc, stimulated the generation of reactive oxygen species (ROS). Hahn et al. (2000) also reported that ROS induction occurred in haemocytes isolated from

snail strains resistant to the trematode infection and also in susceptible strains. Moreover, Castillo and Yoshino (2002) demonstrated that among the simple sugars tested (arabinose, L-fucose, b-lactose, D-mannose, D-mannose-6-P, N-acetyl-D-galactosamine and GlcNAc) only D-mannose-6-P significantly inhibited the adhesion of the Bge to *S. mansoni* primary sporocysts. In contrast, our data showed that the adhesion of circulating haemocytes from *B. tenagophila* to *S. mansoni* sporocysts was blocked by the addition of GlcNAc, but not by mannose and the effect was observed only with haemocytes from the resistant snail strain, *B. tenagophila* Taim.

In conclusion, these data indicate that GlcNAc moieties from glycoconjugates participate in the recognition and subsequent killing mechanism of primary schistosome sporocysts by haemocytes of *B. tenagophila* Taim. The ability of *B. tenagophila* Taim to recognise GlcNAc glycoconjugates from the sporocyst tegument could be involved in snail resistance against *S. mansoni* infection.

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