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QUANTIFICATION OF THE POPULATION AND PHAGOCYTARY ACTIVITY OF HEMOCYTES OF RESISTANT AND SUSCEPTIBLE STRAINS OF *Biomphalaria glabrata* AND *Biomphalaria tenagophila* INFECTED WITH *Schistosoma mansoni*

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SUMMARY

Among the determinant factors in the resistance and susceptibility of *Biomphalaria* to *Schistosoma mansoni*, hemocytes play an important role. Aiming at studying *S. mansoni/Biomphalaria* interactions related to hemocytes, the first step is certainly connected with the standardization of this cell population in uninfected *Biomphalaria*. In this way, quantification of this cell population in hemolymph, as well as its phagocitary capacity, have been determined for the first time. Furthermore, using susceptible and resistant strains of *B. glabrata* and *B. tenagophila*, the hemocytegram and phagocytary capacity of hemocytes after infection with *S. mansoni* were determined too. Resistant and susceptible strains of *B.glabrata* (BA and BH, respectively), as well as resistant and susceptible strains of *B. tenagophila* (TAIM and CF, respectively) were infected with 10 miracidia of the LE and SJ strains of *S. mansoni*, respectively. These infected snails and respective uninfected controls were assessed in relation to the number of circulating hemocytes and alteration in the phagocytary capacity, by using Zymozan and MTT. Reading was taken by means of a spectrophotometer at 5 hours and 1,2,5,10,20 and 30 days after infection. The results showed a decrease in population of the circulating phagocytary cells, 5 hours after infection. One day post-infection, the circulating cells of the susceptible snails showed an increased metabolic activity, but the same event could not be observed in the resistant strains. In the subsequent observation periods, significant differences among the strains studied could not be observed until the end of the experiment.

KEYWORDS: *Schistosoma mansoni*; Hemocytes; *Biomphalaria*, MTT.

INTRODUCTION

Have been demonstrated differences in the susceptibility to infection by *Schistosoma mansoni*, when *Biomphalaria* snails from different regions encounter certain strain of the trematoda [1,2,,8,9,12,26].

The hemocytes - as the main cells of the snail's defence system - prevent dissimilar behaviours regarding schistosome infection, taking into account susceptible and resistant snails.

There is a close similarity between those cells and circulating mononuclear ones (monocytes) of the mammals. They are essential in relation to the recognition, killing and elimination of the invading pathogens [3,4,5,6,17,19]. *Biomphalaria glabrata* - *S. mansoni* has been the most thoroughly studied model, aiming at a better understanding of the mechanisms involved in the susceptibility and resistance in molluscs (gastropoda), in the presence of trematodes [10,11,13,14,18,20,21].

In Brazil, *B. glabrata* is the most important snail vector of *S. mansoni* in the various endemic areas of the country, showing high infection rates, either in nature or experimentally [2,4]. In the South and Southeastern regions of Brazil, *Biomphalaria tenagophila* - although less susceptible to infection by the trematoda than *B. glabrata* - is of great importance in the epidemiology of schistosomiasis [25,26].

Therefore, studies on susceptibility and resistance to *S. mansoni* of strains belonging to different species of the genus *Biomphalaria*, regarding the geographic strains of the parasite,

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are of paramount importance since, from this point on, it would be possible to foresee the potential of a certain area to become endemic.

The scope of this work was to quantify the number of hemocytes in resistant and susceptible strains of *Biomphalaria*, as well as their phagocytary activity pre and post-*schistosome* infection. For this purpose, we used two strains of *B. glabrata*: a susceptible strain (BH strain), and another partially resistant (BA strain). Both strains were infected with the LE strain of *S. mansoni*. Two strains of *B. tenagophila* were equally used: a susceptible strain (CF strain), and another (TAIM strain), resistant. Both *B. tenagophila* strains were infected with the SJ strain of *S. mansoni*.

MATERIALS AND METHODS

Snails and parasites

Two Brazilian strains of *B. glabrata*: BH (susceptible to the LE strain of *S. mansoni*) and BA (partially resistant, to the *S. mansoni* strain from Salvador, State of Bahia), as well as two Brazilian strains of *B. tenagophila*: CF (susceptible to the SJ strain of *S. mansoni* from Paraíba Valley, State of São Paulo) and TAIM (resistant to the *S. mansoni* strain of a biological reserve situated in the extreme South of Brazil, in the State of Rio Grande do Sul), were used in this study. The LE strain of *S. mansoni* was isolated from feces of a patient in Belo Horizonte, MG, Brazil, and has been maintained through successive *B. glabrata* - hamsters (*Mesocricetus auratus*) passages, for more than 30 years. The SJ strain was isolated from naturally infected snails, coming from the Paraíba Valley, SP, Brazil, and has been maintained through *B. glabrata* - *B. tenagophila* - hamsters (*M. auratus*) passages, for more than 17 years. All the snails and parasites have been maintained at the laboratories of the Schistosomiasis Research Unit, Institute of Biological Sciences, Federal University of Minas Gerais, Brazil.

Samples of hemocytes and cell counts

The shell of the snails was cleaned with alcohol (70%), and dried with absorbent paper. After that, it was perforated near the hepatopancreas. The overflowing hemolymph was then collected. So, we got a pool of hemolymph from 5 snails of each species, reaching a 12-15mm volume. The hemolymph so obtained was placed into Eppendorf tubes, left for five minutes, until deposition of shell fragments in the bottom. The hemolymph was then transferred to hemolysis tubes, with a Pasteur pipette, and centrifugated at 100g/10 min. The cells were separated and suspended in 1 ml of Minimum Essential Medium Eagle (MEM, Sigma catalogue no. M-0769). Cell counts were performed as follows: stained (phagocytary) and non-stained (not phagocytary) cells were counted with Newbauer camera, by using neutral red (Sigma catalogue no. 4639) and MEM, since this dye is able to colour only living cells with phagocytary activity. The cell viability was determined by means of Trypan blue incorporation. All the material used in the laboratory was previously siliconized.

Infection of snails and period of analysis

The snail lineages were individually infected with the respective strains (10 miracidia/snail). Miracidia were obtained from eggs in the liver of infected hamsters, as previously described [27].

The hemolymph was collected for analysis, 5 hours and 1,2,5,10,20 and 30 days post-infection.

Assessment of the cellular activation of hemocytes

The same amount of cells was used in all the experiments (1 x 105/100 ml); 40 ml Zymozan (Sigma catalogue, no. 2-4250) were added to hemolysis tubes, to which 600 ml MEM, pH 7.30, were added too, in order to fulfill the volume. After that, the tubes were incubated in water-bath at 37°C, for 30 min. Then, 20 ml MTT (3-[4.5-dimethylthiazol-2yl]-2.5-diphenyl-terazolium bromide (Sigma catalogue no. M-2128) were placed into the tubes, which were then incubated for an additional 2 hr. The temperature of 37°C was chosen because it could allow to stablish an analogy between the phagocitary behavior of hemocytes and phagocitary cells in the definitive hosts of *S. mansoni*. On the other hand, in a simultaneous experiment (report is now in progress), the hemocytes submitted to the temperatures of 15°C, 25°C and 37°C showed an increased phagocitary activity at 37°C.

When MTT (a tetrazolium salt) is reduced, it produces crystals of formazan (of chestnut brown-bluish colour), which can be solubilized with isopropanol-HCl. The colouration intensity is proportional to the number and metabolic conditions of the cells. Reading was taken by means of spectrophotometry at 570 nm. The control group of the cell under its basal conditions was composed of cell, culture medium and MTT. NUÑEZ et al.[24] have carried out studies on hemocytes from *Lymnea stagnalis*, by using MTT too.

Two groups were used for all the experiments in this study: infected and uninfected groups, in the presence or absence of Zymozan. All the experiments were carried out twice.

Statistical analysis

The mean and standard deviation were determined aiming at calculating the number of phagocitary cells. The analysis of variance (one-way) and the Student's t test were used for comparison in the infection periods.

RESULTS

Hemocytegram

Determination of the number of phagocytary and non-phagocytary cells of the analyzed strains (uninfected) was carried out by means of staining, i.e., the phagocytary cells were coloured by neutral red. Proportion of staining (phagocytary) and non-staining cells present in the hemolymph of uninfected snails did not differ quantitatively between the species and strains of *Biomphalaria* studied (Table 1).

TABLE 1

Quantification of phagocytary and non-phagocytary cells present in the hemolymph of two strains of *B. glabrata* and in two other ones of *B. tenagophila*, by using the neutral red.

NO INFECTED SNAIL	PHAGOCITARY CELLS	NO PHAGOCITARY CELLS
B. glabrata (BH)	9.74 ± 1.02 (81.2%)	2.25 ± 0.46 (18.7%)
B. glabrata (BA)	10.20 ± 0.75 (81.9%)	2.00 ± 0.25 (18.1%)
B. tenagophila (CF)	9.21 ± 0.57 (81.0%)	2.16 ± 0.40 (19.0%)
B. tenagophila (TAIM)	8.98 ± 0.86 (81.2%)	2.08 ± 0.30 (18.8%)

Evaluation of the hemocytes function by their phagocytary activity

As can be seen in Fig. 1, a statistically significant decrease (Student's t test, $P < 0.03$) of circulating hemocytes in the four studied lineages could be observed, 5 hr post-infection. However, as showed in Figs. 2A and 2B, such decrease of circulating hemocytes could not be detected on 1,2,5,10,20 and 30 days after infection. Fig. 3 shows a statistically significant cellular activation (analysis of variance, $P<0.01$), determined by means of MTT and Zymozan, regarding hemocytes of the susceptible strains (BH and CF), within a 24 hr-period after infection, that activation being not observed after 48 hr post-infection. Nevertheless, subsequent observations were not able to detect significant differences among the studied strains, till the end of the experiments.

DISCUSSION

In Brazil, schistosomiasis is one of the most important endemic diseases affecting millions of people. The snails belonging to the genus *Biomphalaria* act as vectors [16].

Among the determinant factors in the resistance or refractoriness of *Biomphalaria* snails regarding *S. mansoni,* the phagocytary cells or hemocytes, and their interaction with hemolymph lectines, are the most relevant elements [10,15,19,20,21,22,28,29,30]. The phenomenon of primary sporocyst encapsulation by hemocytes (amebocytes) of resistant strains has been discussed by several authors[6,8,17]. On the other hand, LOKER et al.[22] demonstrated the existence of lectines, that they named agglutinins (opsonines). These agglutinins, that bind to the wall of primary sporocysts of *S. mansoni*, were present in two resistant strains of *B. glabrata*, and absent in three susceptible ones of the same species. It was verified that lectines promote the connection between hemocyte/sporocyst, when binding to carbohydrate of cellular walls of hemocytes and primary sporocysts, thus beginning a process of endocytosis[3]. *B. glabrata* snails (genetically resistant or susceptible to *S. mansoni*) presented differential expression of lectines [29]. Recently, MANSOUR[23] demonstrated in *Biomphalaria alexandrina* the presence of two types of isolectine, which could play an important role in the hemocyte/parasite interaction.

The present study was undertaken by using geographical strains of the two most important species in the transmission of schistosomiasis mansoni in South America and, practically, there are no studies on cellular and humoral mechanisms that rule the susceptibility or resistance of these South-American molluscs regarding *S. mansoni.*

The initial step for this kind of study was the quantification of circulating hemocytes in hemolymph (hemocytegram), as well as determination of their phagocytary capacity in normal snails (uninfected). Table 1 shows a close similarity between the number of circulating hemocytes and the proportion of phagocytary cells among the strains of the studied species.

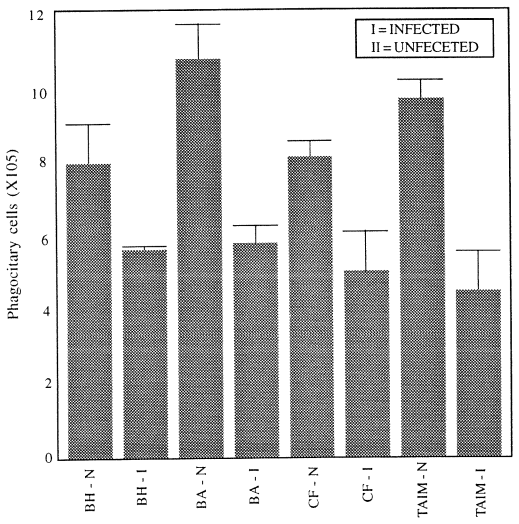

Fig. 1- Number of phagocitary cells (Mean± S. D.) *of B. glabrata* (BH and BA) and *B. tenagophila* (CF and TAIM) infected with *S. mansoni* and examined 5 hours later with their respective uninfected controls.

The infection by *S. mansoni* showed, as far as the infection period is concerned, a significant decrease of circulating hemocytes after 5 hr, in all the observed strains (Fig. 1). Further, a marked decrease of circulating hemocytes in the resistant strains could be observed (Fig. 3). These results could be interpreted as the way of circulating hemocytes toward the tissular region,

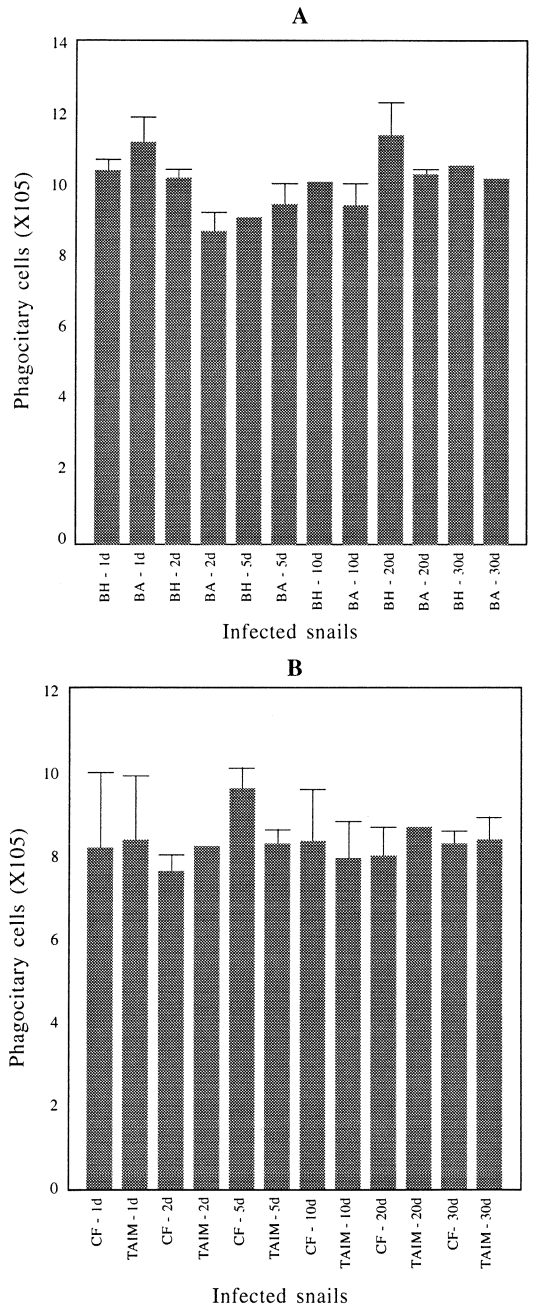

Fig. 2- Number of Phagocitary cells (Mean± S. D.) of the snails infected with *S. mansoni* and examined 1, 2, 5, 10, 20 and 30 days after infection. (A) *B. glabrata* (BH and BA). (B) *B. tenagophila* (CF and TAIM).

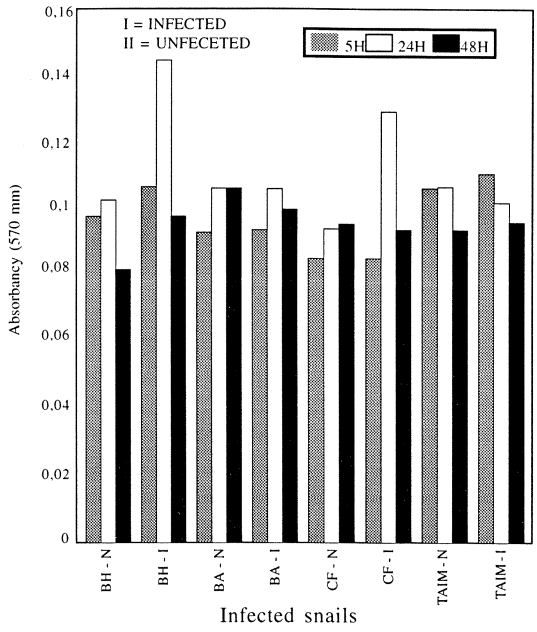

Fig. 3 - Evaluation of phagocytosis of Zimozan particles by hemocytes from *B. glabrata* (BH and BA) and *B. tenagophila* (CF and TAIM), and measured by MTT metabolization at 5, 24 and 48 hours after infection with *S mansoni.* and yours respective controls.

where the sporocyst was settled down. The result obtained related to the increase of cellular activation determined by means of MTT, in the presence of a stimulus (Zymozan), that was observed in the susceptible strains of both species within a 24 hr-period post-infection, could be explained by the absence of certain lectines in these strains, that could promote a more firm connection between saccharides of the membrane of hemocytes and those ones of the membrane of sporocysts. Thus, the cells could be activated by contact with the parasite, but they would not have the encapsulation ability of the parasite, that occurs only in the resistant strains, returning to circulation, and their activation could be maintained for a minimum period of 24 hr after infection. This cellular activation could not be detected 48 hr after infection (Fig. 3). The phenomenon of cellular activation being maintained for 24 hr, and disappearing after 48 hr, has some analogy with the behaviour of the mononuclear cells of mammals[7]. These results reinforce the most consistent hypothesis formulated by specialists on this matter, i.e., that the destiny of schistosome infection in the intermediary host is defined in the first moments after miracidium penetration[10]. Finally, studies aiming at elucidating the mechanisms that govern the susceptibility or resistance to *S. mansoni* of geographic strains of *Biomphalaria* (with epidemiological importance) are needed, since from the knowledge of the intrinsic processes of a certain geographic strain onwards it could be possible to foresee its potential as vector of schistosomiasis mansoni.

RESUMO

Quantificação da população e atividade fagocitária de hemócitos de cepas resistentes e suscetíveis de *Biomphalaria glabrata* e *Biomphalaria tenagophila* infectadas com *Schistosoma mansoni*

Entre os fatores determinantes na resistência e suscetibilidade da *Biomphalaria* ao *Schistosoma mansoni,* os hemócitos

desempenham importante papel. Com o objetivo de estudar as interações *S. mansoni/Biomphalaria* relativas aos hemócitos o primeiro passo é certamente relacionado à padronização desta população de células em *Biomphalaria* não infectada. Desta forma a quantificação desta população de células na hemolinfa bem como sua capacidade fagocitária foi determinada pela primeira vez. Além disso, usando cepas de *B. glabrata* e *B. tenagophila* suscetíveis e resistentes, o hemocitograma e a capacidade fagocitária dos hemócitos, após infecção com *S. mansoni,* foram também determinados. Cepas de *B. glabrata* (BA e BH respectivamente) resistentes e suscetíveis bem como cepas de *B. tenagophila* (TAIM e CF respectivamente) foram infectadas com 10 miracídios das cepas LE e SJ de *S. mansoni*, respectivamente. Estes caramujos infectados e respectivos controles não infectados foram avaliados em relação ao número de hemócitos circulantes e alteração da capacidade fagocitária, usando Zimozan e MTT. A leitura foi feita por meio de espectrofotômetro com cinco horas e 1,2,5,10,20 e 30 dias após a infecção. Os resultados mostraram diminuição na população de células fagocitárias circulantes, cinco horas após a infecção. Um dia após a infecção as células circulantes dos caramujos suscetíveis mostraram aumento da atividade metabólica, mas o mesmo fato não pode ser observado nas cepas resistentes. Nos períodos de observação subseqüentes, diferenças significantes entre as cepas estudadas não puderam ser observadas até o final do experimento.

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