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Artículo:

Morfología de la superficie y características de los hemocitos de *Biomphalaria glabrata* (pulmonata: planorbidae) de dos procedencias geográficas

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ORIGINAL ARTICLE

Surface morphology and characteristics of hemocytes of *Biomphalaria glabrata* (Pulmonata: Planorbidae) from two geographic sources

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ABSTRACT. *Biomphalaria glabrata* is a fresh water snail of medical importance since it is the intermediate host of the trematode *Schistosoma mansoni*. The internal defense system of mollusks is mostly represented by circulating elements of the hemolymph (hemocytes). The infectivity of *S. mansoni* to *B. glabrata* is determined by genetic factors and evolutionary adaptations. In the present work factors about the parasite/snail relationship were evaluated, especially those related to the morphology and characteristics of the surface of cells present in the hemolymph of two strains of *B. glabrata*: a strain with high susceptibility to S. mansoni (Puerto Rico, PR) and a strain with medium susceptibility (Caripe, Ca). Hemolymph was collected by cephalopodal puncture; a total and a differential count of hemocytes were done in dyed preparations and through scanning electron microscopy (SEM). Results from both strains show a high quantitative variability of the total hemocyte count. Hemocytes dyed with MayGrünwald Giemsa showed cells with a basophilic nucleus predominantly in PR (61.7%), dense and strongly basophilic in Ca (71.2%) with significant differences between them. Through SEM round cells with a corrugated surface were observed (6-10µm), hemocytes with an irregular spongy surface (12µm), others with many projections (16µm) and cells not reported in similar to erythrocytes (21µm). Hemocytic cells from both strains confirmed cytoadherence and encapsulation were confirmed in the hemocytic cells from both strains, while no differences associated to the susceptibility of the strains were observed after 2 h of parasite-hemocyte incubation

Key words: Hemocytes, hemolymph, *Biomphalaria glabrata, Schistosoma mansoni,* scanning electron microscopy.

RESUMEN. *Biomphalaria glabrata* es un caracol de agua dulce de importancia médica por ser el hospedador intermediario del tremátode *Schistosoma mansoni*. El sistema de defensa interno de moluscos está representado fundamentalmente por elementos circulantes (hemocitos) de la hemolinfa. La infectividad de *S. mansoni* a *B. glabrata* está determinada por factores genéticos y adaptaciones evolutivas. En el presente trabajo se evaluaron factores de la relación parásito/caracol, especialmente relacionado a la morfología y las características de la superficie de las células presentes en la hemolinfa de una cepa de *B. glabrata* de alta (Puerto Rico, PR) y otra de mediana (Caripe, Ca) susceptibilidad a *S. mansoni*. La hemolinfa fue colectada mediante punción en la región cefalopodal, se hizo recuento total y diferencial de los hemocitos en preparaciones teñidas y por microscopía electrónica de barrido (MEB). Los resultados de ambas cepas muestran una alta variabilidad cuantitativa de los hemocitos totales. Los hemocitos teñidos con MayGrünwald Giemsa mostraron predominancia de células con núcleo basófilo en PR (61.7%), denso y fuertemente basófilo en Ca (71.2%) con diferencias significativas entre sí. Por MEB se observaron células redondeadas con superficie rugosa (6-10µm), hemocitos de superficie irregular y aspecto esponjoso (12µm), otra con numerosas proyecciones (16µm) y células no reportadas en *B. glabrata* con aspecto semejante a eritrocitos (21µm). En las células hemocíticas de ambas cepas, se confirma la citoadherencia y encapsulación sin observar, a las 2 h de incubación parásito-hemocito, diferencias asociadas a la susceptibilidad de las cepas de caracol.

Palabras clave: Hemocitos, hemolinfa, *Biomphalaria glabrata, Schistosoma mansoni,* microscopía electrónica de barrido.

INTRODUCTION

Biomphalaria glabrata is a fresh water planorbidean mollusk of medical interest since it is the intermediary host of *Schistosoma mansoni,* a trematode parasite that inhabits the blood stream of the infected person, causing schistosomyasis.[7,21] The natural populations of the mollusk, which come from different geographic sources, present a variable degree of susceptibility to *S. mansoni,*[9,13] assuming that there are differences in the genetic composition among different populations of *B. glabrata*.[5, 25] At the same time, isolated *S. mansoni* from different regions varies in its infectivity for a particular strain of mollusk, suggesting that the relation *S. mansoni*-invertebrate host is influenced by co evolution dependent factors.[15]

Studies about the molecular and cellular bases of the susceptibility or resistance of *B. glabrata* to *S. mansoni,* indicate that hemocytes and humoral factors are determinant for the success of the infection.[8,12,13, 22]

With the purpose of studying other factors in the parasite-invertebrate host relationship, we compared morphology, types and aspects of the surface of *B. glabrata* hemocytes from two different sources and susceptibility to infection by *S. mansoni*.

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MATERIAL AND METHODS

Mollusk management. Sub-adult snails were selected[30] (7 - 10 µm diameter). *B. glabrata* from the Caripe (Ca) and Puerto Rico (PR) strains were kept in glass containers in chlorine free water, adequate aeration and fed with fresh lettuce *ad libitum*.

***S. mansoni* miracidia obtention.** Eggs were obtained by enzymatic digestion of hamster livers with 8 weeks of infection, having 800 to 1000 *S. mansoni* (strain PR)[4] cercariae (cercarias). The purified eggs were placed in sterile distilled water, under direct light to favor eclosion and liberation of miracidia, which were then collected and cold concentrated.

Snail hemolymph obtention. Each snail was cleaned externally with isopropanol and transferred to a container with sterile distilled water supplemented with antibiotics (penicillin 1000 U and streptomycin 500 µg/ml). After 12 h, they were anesthetized by immersion in urethane at 1.5% during 1 h at room temperature. To obtain the hemolymph, a puncture in the cephalopodal region was performed to groups of 20 snails; the hemolymph was kept in an ice bath during its collection.

Hemocyte count and dyeing. 10 µl of hemolymph were placed directly into a Neubauer chamber (camara) to do a cell count at 400X.[11,31] Hemolymph mixtures from each group of both strains were placed on 22 x 22 mm slides; thy were dried at room temperature, fixed with methanol and dyed with MayGrünwald-Giemsa. Observation was done by immersion into an Eclipse 400, Nikon photomicroscope.

Table 1. Total hemocyte median in 20 healthy *Biomphalaria glabrata* snails, Puerto Rico and Caripe strains.

Strain	Hemocyte count/µl		
	Median (x ± s.d.)	minimum	maximum
Puerto Rico	1,731.8 ± 1,476.1[1]	400	6,450
Caripe	2,077.6 ± 1,631.6	450	6,500

[1] p<0.005 non-significant

Table 2. Differential count median of the hemocytes of 20 healthy *B. glabrata* snails, strains Puerto Rico and Caripe.

Strain	Hemocyte types (%)			
	I*	II*	III*	IV
Puerto Rico	61.7 ± 6.8[1]	22.1 ± 11.1	9.8 ± 4.1	6.3 ± 5.0
Caripe	21.3 ± 13.9	71.2 ± 17.3	1.6 ± 3.5	7.1 ± 7.4

[1] arithmetic median ± s.d.

* p < 0.005 significant.

Scanning electron microscopy. Fresh hemolymph was fixed with glutaraldehyde at 2% (v/v) in Millonig 0.2 M; pH 7.3 buffer solution, washed in buffer, post-fixed with osmium tetraoxide at 1% (p/v) and placed in dehydration slides with ethanol and amilum acetate, followed by a critical point desiccation process with CO_2 and a platinum/palladium covering. Observation was done using a Jeol model 5300 scanning electron microscope.

Hemocyte adherence to glass and interaction with miracidia. Snails were placed in a 1:10,000 thiomersal solution in sterile distilled water during 5 min, in sterile distilled water without thiomersal for 10 min and finally they were washed 6 times in water with antibiotics for 20 min. 10 µl of hemolymph obtained through cephalopodal puncture out of 20 snails were placed 22 × 22 mm slides in a humid chamber for 2 h at 27°C.[1,3,17] The supernatant was discarded with the hemocyte that did not adhere to the glass. The adhered population was exposed to 150 *S. mansoni* miracidia at a volume of 25 µl for 1h at 27ºC. The adherence of the hemocytes and their interaction with the miracidia was evaluated in an inverted microscope, brand Zeiss, model ID3.

Statistical process. Total and differential counts of the dyed hemocytes were compared using a Student t test, for P< 0.005.

RESULTS

The count of circulating hemocytes in the hemolymph from the isolates Ca and PR of *B. glabrata* is shown in Table 1. Quantitative variation of hemocytes was observed (400 at 6,500/µl) (400 to 1) with a median of 1,731 hemocytes in PR and 2,077 in Ca, with out statistically significant differences between them.

Dyeing of the hemocytes with May Grünwald-Giemsa was better than with Giemsa & Wright[13] (Fig. 1), and the following cell types were identified: type I, basophilic nucleus hemocytes with dense chromatin and basophilic cytoplasm (Fig. 1A); type II, with a basophilic nucleus light chromatin and lightly basophilic and cytoplasm with vacuoles (Fig. 1B); type III, spherical, with a large nucleus, little cytoplasm and uniformly basophilic.; (Fig 1C) and type IV, spherical anucleated, uniformly basophilic and granular (Fig. 1D).

Table 2, summarizes the differential count of hemocytes in the hemolymph of *B. glabrata*. In the PR strain, type I hemocytes were predominant (61.7%) together with type II hemocytes (22.1%), adding up to 83.8% of the total circulating hemocytes; while the Ca strain the percentage of these two types is inverted with type I (21.3%) and type II (71.2%) adding up to 92.5% of the total circulating hemocytes. Comparison between the cell types in both strains showed statistically significant differences in types I, II and III.

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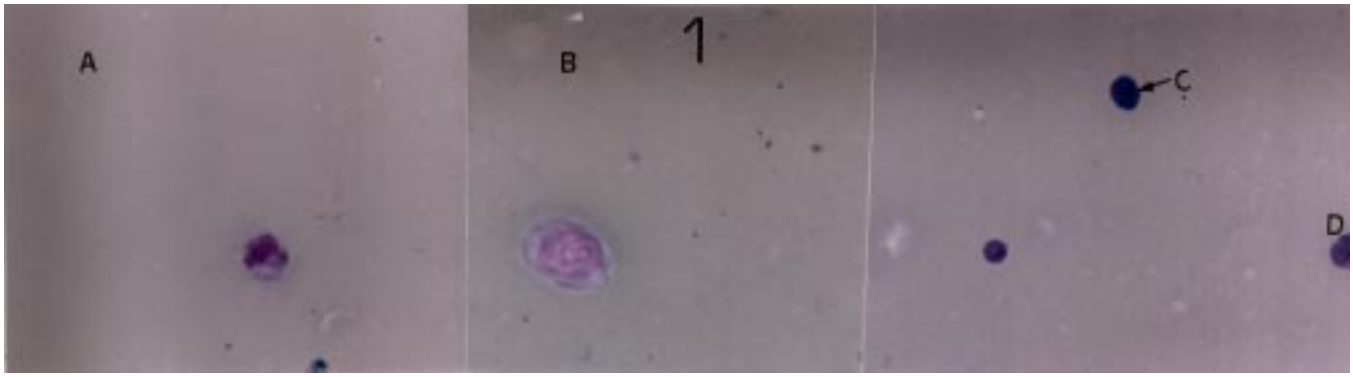

Figure 1. Biomphalaria glabrata *PR hemocytes dyed with May Grünwald-Giemsa: A) Type I, basophilic nucleus and dense chromatin; B) Type II, basophilic nucleus and lax chromatin lax; C) Type III, spherical large nucleus, low cytoplasm, uniformly basophilic; D) Type IV, spherical, anuclear, uniformly basophilic and granulated. Augmentation 1000x.*

The adherence to glass assay revealed, for both strains, hemocytes with type II characteristics with thin cytoplasmic prolongations and type III at a lower proportion. The adherent hemocytes from strains Ca and PR, showed a similar interaction pattern with the miracidia of *S. mansoni* (encapsulation) (Fig. 2).

Using SEM, round cells with projections on their surface were identified on both strains in strain PR (Fig. 3A). For strain Ca, similar cells presented an irregular surface that simulates microfollicles or cellular secretion product deposits (Fig. 3B). Also, other cells were observed: 12 µm round cells with an irregular surface and spongy in appearance (Fig. 3C), 6-12 µm corrugated surface round cells that were smaller than the latter (Fig. 4A), and biconcave cells similar to erythrocytes, of approximately 21 µm (Fig. 4B).

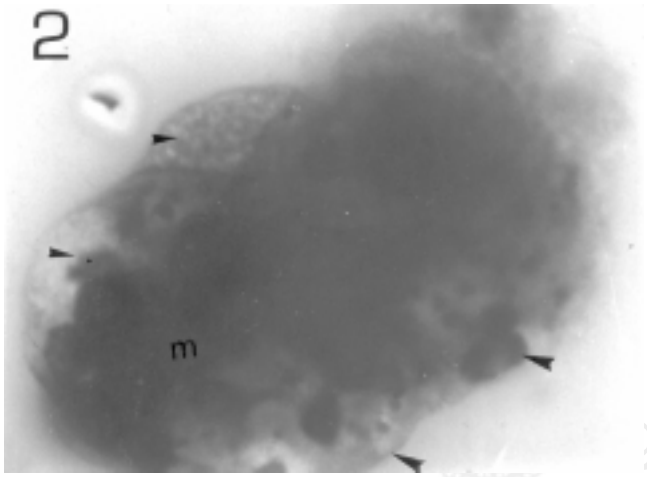

Figure 2. Biomphalaria glabrata *PR hemocytes engulfing a* Schistosoma mansoni *miracidium after one hour of incubation. Augmentation: 1000x.*

DISCUSSION

The susceptibility of fresh water mollusks of genre *Biomphalaria* to infection by *S. mansoni*, is associated to the hemocytes present in the hemolymph.[10,29] These cells present a morphological and biochemical heterogeneity, that makes their characterization difficult, based on simple criteria: for example specific secretion granules that are present in other invertebrates.[14,22] This originates two possible explanations: the existence of a single cell type at different stages of maturity, or several sub-populations with specific functions.[23] The present work compared to isolated strains of *B. glabrata* with different origin and susceptibility to *S. mansoni*, observing a great variability in the number of circulating hemocytes in each of the mollusk's populations (400 to 6,500). Similar observations are reported by Barraco y col.[2] in adult mollusks, but of the *B. tenagophyla* species. In addition, other authors point out that the number of hemocytes in the hemolymph is influenced by the snail's age, physiological condition and by the method of obtention[8,9, 27] which suggests that cell quantification has little value in the characterization of mollusk populations.

Hemocytic cells are not easily colored, and a better contrast is obtained with MayGrünwald-Giemsa. Four types of hemocytes were observed in both isolates. These results contrast with those of other authors, which only describe two types of hemocytes in the hemolymph.[5-7,16] Barraco & col.[2] report, in *B. tenagophyla*, two populations of hemocytes, hyalinocytes hialinocitos) (large nucleus and basophilic cytoplasm) and granulocytic cells (large cells, small nucleus, and high cytoplasmic content) in a proportion higher than 90%. According to the classification proposed in this work, type III cells correspond to the "hyalinocytes", and types I and II to the "granulocytes".[2] Other studies of the species *B. glabrata*, *B. truncatus* and *Lymnea stagna-*

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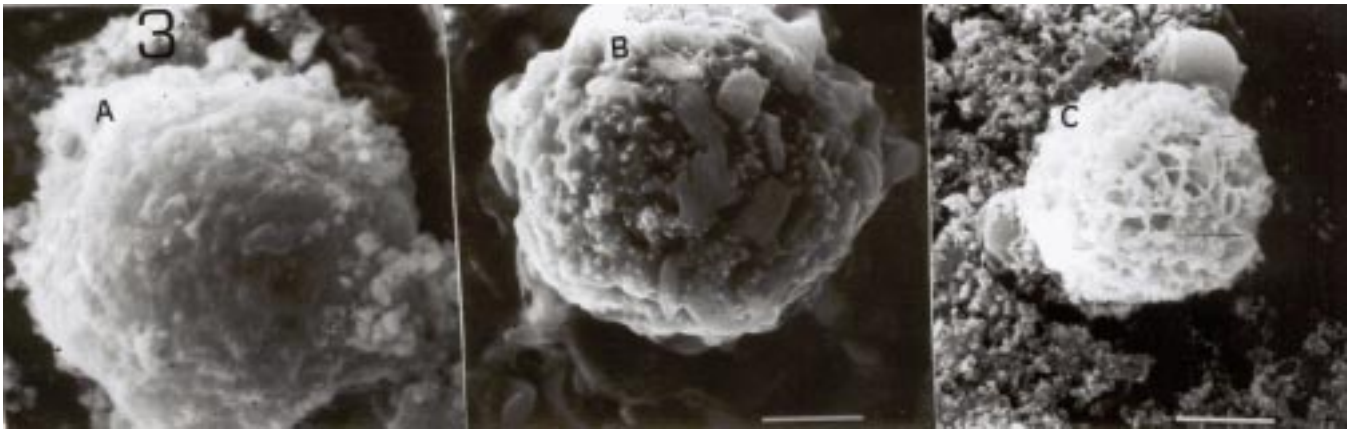

Figure 3. *Scanning electron microscopy of* Biomphalaria glabrata *hemocytes: A)* B. glabrata *PR hemocyte, round with surface projections. Bar = 1 µm; B)* B. glabrata *Ca hemocyte with surface particles similar to microvelli or secretion point. Bar = 5 µm; C) Present in both strains, a hemocyte with an irregular surface and spongy in appearance Bar = 5 µm.*

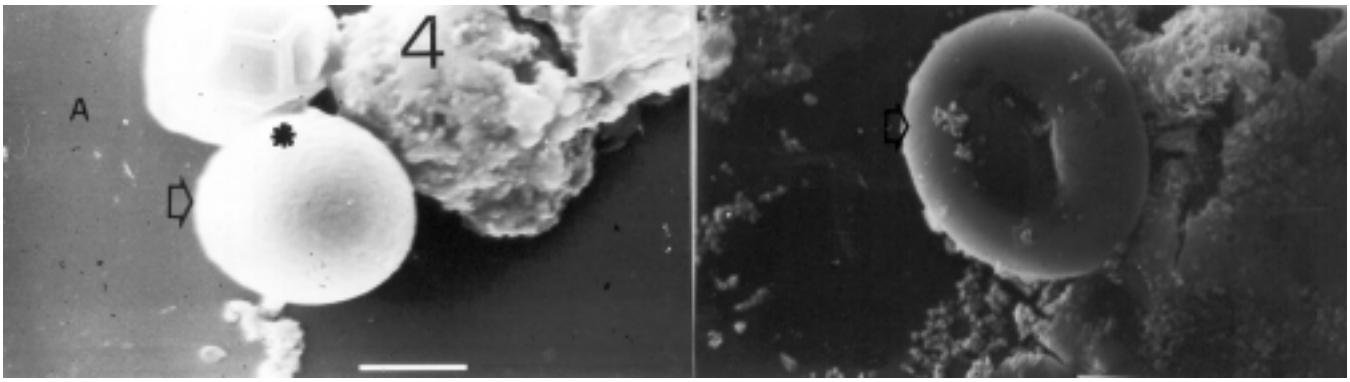

Figure 4. *Scanning electron microscopy of* Biomphalaria glabrata *hemocytes in strains Ca and PR: A) Round hemocyte with corrugated surface. Bar = 5 µm; and B) Hemocyte with a morphology similar to an erythrocyte. Bar = 10 µm.*

lis,[28] describe two morphologically different populations of hemocytes: one formed by round cells and another by hemocytes that project cytoplasmic elongations. The discrepancy in the hemocyte type characterization could be due two the difficulties in coloration that these cells present and to the fact that the majority of these works describe adherent cells. It is worth mentioning that we do not consider appropriate the use of some authors of the term "granulocytic", since these cells do not possess peroxidase type granules, as it has been described in vertebrates and invertebrates.[15] For this reason we adopted the general definition of hemocyte and differentiated four types of hemocyte on the bases of morphology and dyeing affinity of the nucleus and cytoplasm out of cells present in the hemolymph from the isolated Ca and PR strains of *B. glabrata*.

The adherence to glass assays show that only hemocytes type II and III have this property, in different compatibility hosts, and the ability of the adherent cells to encapsulate *S. mansoni* miracidia, in 2 h of contact was similar for both strains. Sapp & Loker[24] report similar results when comparing 31 combinations different compatibility degrees during one hour of contact suggesting that the relation hemocyte-miracidium is not an incompatibility indicator, at least for the examined time period. Other studies conclude that the encapsulation process and the production of toxic radicals against the parasite define resistance.[29,32] The fact that no differences were observed in the adherence and encapsulation results of hemocytes from the PR and Ca strains, suggests a need for longer interaction studies to make evident the differences in the snail strains reported by other authors.[18-20]

The SEM showed concordance to the types of hemocytes observed in the light microscope, except for the cells that looked similar to erythrocytes (type IV), which constitutes a new finding in the present work. The existence of

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these cells is supported by the results of Stang Voss,[29] which describe erythrocyte type cells in the connective tissue of *L. stagnalis* with the purpose of forming and storing pigments. Given the lack of more information in relation to this cell type, we recommend its study through the use of a transmission electron microscope, which would help determining the presence of a nucleus and characteristics proper to erythrocyte type cells found in other mollusks.

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