ISJ 15: 346-351, 2018 ISSN 1824-307X

SHORT COMMUNICATION

# Hemocytes of mollusc *Biomphalaria glabrata* (Gastropoda, Pulmonata)

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*Accepted September 28, 2018*

## Abstract

We studied hemocytes of *Biomphalaria glabrata* with the use of light and transmission microscopy and flow cytometry with LysoTracker and SYTO62. These specific fluorescent dyes make it possible to distinguish cells differing in the amount of lysosomes and in transcriptional activity. We found that hemocytes of *B. glabrata* were represented by hyalinocytes and granulocytes (35.34 (27.38; 36.10) and 65.13 (64.11; 73.05) % respectively). Granulocytes were generally strongly granulated and more metabolically active than hyalinocytes. This is the first report confirming the presence of these two main cell types in the hemolymph of *B. glabrata* with the use of specific fluorescent dyes.

**Key Words**: *Biomphalaria glabrata*, hyalinocytes, granulocytes, flow cytometry, LysoTracker, Syto62

## Introduction

Molluscs *Biomphalaria glabrata* are broadly used in physiological, parasitological and comparative immunological studies. An interest in them is mainly due to their role as intermediate hosts in the life cycles of trematodes, some of which, such as schistosomes, are dangerous human pathogens. Defence reactions of *B. glabrata* have been studied intensively for decades. As in other gastropods, the main component of their immune response are hemocytes, the cells of the hemolymph. Morphology, functional activity and origin of hemocytes have been described in numerous studies (Yoshino *et al.,* 2013; Pila *et al.,* 2016; Zhang *et al.,* 2016). Nevertheless, even the number of types of these cells remains a debated question. While most authors consider that *B. glabrata* has two types of hemocytes, hyalinocytes and granulocytes, some authors have reported four and more types (Joky *et al.,* 1983; Delgado *et al.,* 2001; Cavalcanti *et al.,* 2012). Obviously, new methods and approaches ought to be employed in further studies of hemolymph cells.

A promising technique of studying circulating cells of different animals is flow cytometry. Differences in cell composition of uninfected molluscs *B. glabrata* and molluscs infected by *Schistosoma mansoni* (Trematoda) and *Angiostrongylus vasorum* (Nematoda) were found with the use of this method (Martins-Souza *et al.,* 2009; Barcante *et al.,* 2012). Flow cytometry has also been used for the study of the hemolymph of *Planorbarius corneus* and *Planorbis planorbis* (Ottaviani and Cossarizza, 1990; Ataev *et al.*, 2016). Flow cytometry with specific fluorochromes makes it possible to define functional characteristics of hemocytes and to verify the validity of their morphological hemocytes populations. However, specific cell dyes have never been used before for *B. glabrata*. In this study we carried out a complex analysis of hemocytes of *B. glabrata* with the use of light and transmission microscopy and flow cytometry with the use of specific fluorescent dyes. The first dye, LysoTracker, accumulates in cell compartments with a lowered pH (MacIntyre nad Culter, 1988). The second dye, SYTO62, is a cationic cyanine dye of nucleic acids (Cosa *et al.,* 2001; Wlodkowic *et al.,* 2009). A change of the fluorescence level in a stained cell may indicate the degree of chromatin condensation and a change in the RNA content in it (Grinchenko *et al.,* 2014). If both dyes are used, the cells can be divided into populations differing as to the amount of lysosomes and transcriptional activity (Kudryavtsev *et al.,* 2012).

## Material and methods

We used snails *B. glabrata* of the laboratory Brazilian strain (Brazil) with a shell diameter of 14-16 mm. The hemolymph was sampled from the blood sinus of the snail's head with the help of a glass Pasteur pipette. The hemolymph was mixed with Chernin's solution (1:1) (Chernin, 1963) to dissolve it and to prevent agglutination of hemocytes.

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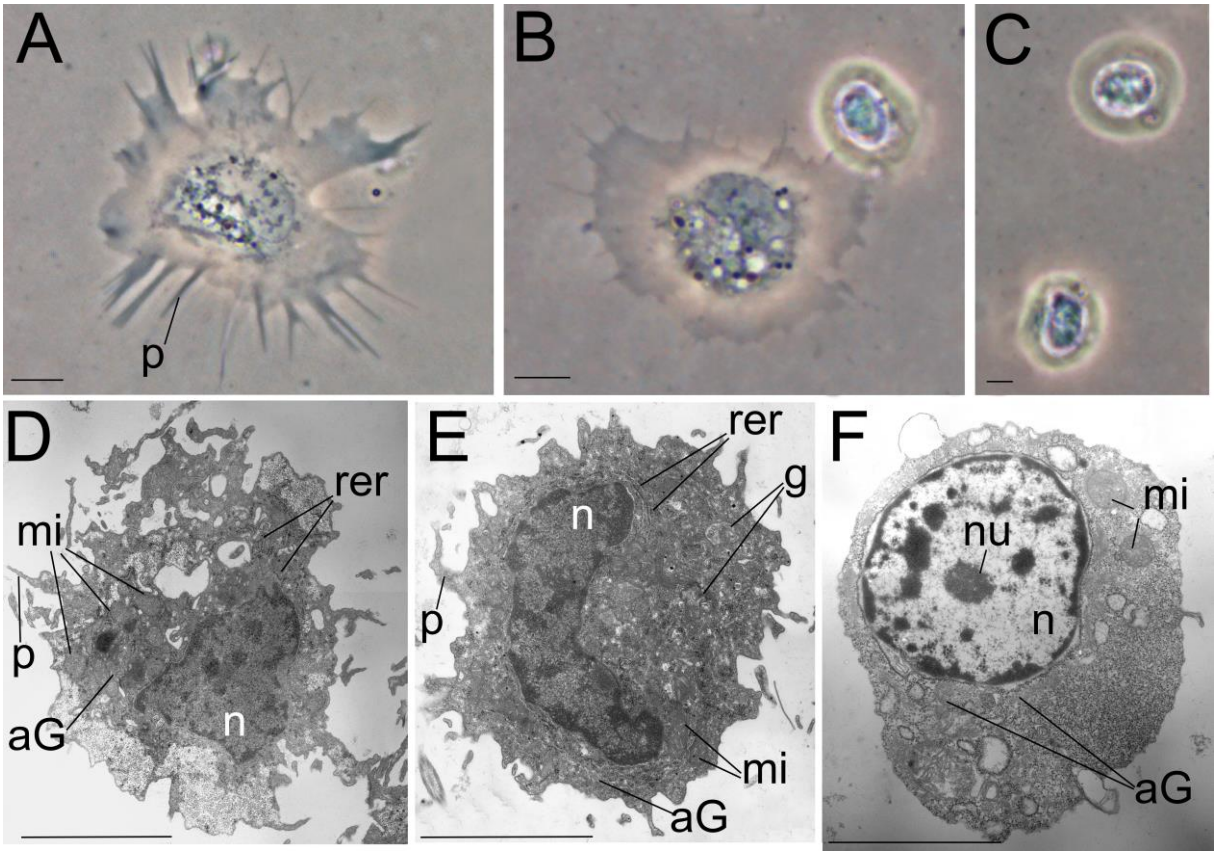


**Fig. 1** Morphological types of hemocytes of molluscs *B. glabrata*. A–C phase contrast image of live cells, D–F – electron microscopic micrograph. A, D, E – granulocytes, C, F – hyalinocytes, B - granulocyte (left) and hyalinocyte (right). Abbreviations: aG –Golgi apparatus, g – granules, mi – mitochondria n – nucleus, nu – nucleosis, p – pseudopodia, rer – rough endoplasmic reticulum. Scale – 2 μm

For *in vitro* morphological analysis of the hemolymph cells, freshly sampled hemolymph from individual snails (n=40) was smeared on Teflon-printed glass microslides (well diameter, 5 mm) and analysed with the use of Leica DM5000 light microscope equipped with the phase contrast. The cells of each snail were counted in cell-counting chamber according to a previously described technique (Ataev *et al.,* 2016). In brief, from 3 to 6 hemolymph samples were collected from every mollusc. Hemocytes in 1 μl of hemolymph were counted in a cell-counting chamber (Cell-Line Associates). For *in vitro* study, hemolymph from individual snails was placed into plastic Petri dishes and incubated in a humid chamber for 4-8 h at 22-24 °C. During incubation the samples were observed intermittently (every 10-20 min) under a phase-contrast microscope (Leica DM 5000). Cell counts and size measurements were made using ImageScope software (CMA, Russia).

Total hemolymph from at least 10 snails was fixed at 4 °C using a solution of 2.5% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.2. The samples were washed in 0.05 M cacodylate buffer, pH 7.2 and embedded in 1.5% low melting point agarose. The resulting agarose blocks were post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h. Finally, the blocks were dehydrated through a graded ethanol series followed by acetone, and embedded in Epon 812. Ultrathin sections (30–50 nm thick) were double-stained with uranyl acetate and lead citrate, and observed using a Jeol JEM 1011 electron microscope.

Hemolymph from individual snails (n=15) for the flow cytometric assay was diluted with Chernin's solution (1:1) (Chernin, 1963) and stained with SYTO62 (SYTO62® Red Fluorescent Nucleic Acid Stain, emission maximum 680 nm) and Lysotracker dyes (LysoTracker® Green DND-26, emission maximum 511 nm) (Prokhorova *et al.,* 2018). Flow cytometer BD Accuri™ C6 (lasers with wavelengths of 488 and 640 nm) (BD Biosciences) was used. For registration of fluorescence FL1 (533±30 nm) and FL4 (675±25nm) detectors were used. For each cell we assessed the forward scatter (proportional to the cell size), the side scatter (characterizing the cell structure) and the fluorescence intensity of the dye (Mullaney *et al.*, 1969; Loken *et al.*, 1976). From 5000 to 10000 events were analysed for each individual haemolymph sample.

Mathematical processing of the data was performed with the help of Kaluza™ v.1.5 (Beckman Coulter, USA) and NovoExpress (ACEA, USA) software. Statistical analysis was performed with the

help of GraphPad Prizm and Microsoft Excel software. The results were represented as the median (Me) and the interquartile range (Q25, Q75). Statistical significance was assessed with the help of non-parametric Mann-Whitney U-test.

To obtain quantitative data about the populational composition of the hemolymph we constructed two variants of two-parameter bar charts: based on the values of the side scatter and the forward scatter and based on the values of the fluorescent unit of SYTO62 and LysoTracker Green dyes. Our approach to the populational analysis of the hemocytes was based on the construction of hierarchic dendrograms. Populations distinguished on the basis of the side scatter and the forward scatter were then separated based on the fluorescence intensity of both dyes (Kudryavtsev *et al.,* 2016).

## Results

Most of the hemolymph cells of *B. glabrata* (n=40) were represented by granulocytes. These were the largest hemocytes (14.03±5.6 µm in diameter, n = 180), which could spread on the substrate. Their nucleoplasmic ratio (NCR) varied from 0.08 to 0.13. These cells may form both filopodia and lobopodia. Their nucleus is eccentric, irregular in shape (7.00±1.2 µm in diameter). It contains visible accumulations of condensed chromatin, mostly lying under the nuclear envelope. The nucleolus was not observed. The cytoplasm is rich in organelles. The centre of the cell is occupied by small dictyosomes, multivesicular bodies, mitochondria and a well-developed RER. Free ribosomes and small glycogen accumulations could be seen in the cytoplasm (Fig. 1).

The second type of hemocytes was represented by hyalinocytes. They are smaller than granulocytes (7.21±1.01 µm in diameter, n = 57). The cytoplasm contains numerous free ribosomes, the Golgi apparatus and a few mitochondria and other organelles. Hyalinocytes never contained accumulations of granules, which were present in granulocytes. The nuclei of hyalinocytes (4.51±1.19 µm diameter) are eccentric, and condensed chromatin is diffused. A nucleolus is well visible in the centre of the nuclei. Hyalinocytes may form infrequent lobopodia but cannot properly spread on the substrate (Fig. 1). Their NCR lies in the range of 0.19-0.27.

According to the results of counts in the cell-counting chamber, one microliter of the *B. glabrata* hemolymph (n=55) contained 138 (107-169) cells. Similar results were obtained with the use of flow cytometry: 141 (median 120.67 (68.29; 172.02) cells/µl.

The cytometric assay showed that there were two hemocyte populations in *B. glabrata* (n=15) (Fig. 2A): cells with lower values of forward scatter and side scatter (FSlowSSlow) and cells with higher values of forward scatter and side scatter (FShighSShigh). The former population corresponded to hyalinocytes (65.13 [64.11; 73.05]), while the latter corresponded to granulocytes (35.34 [27.38; 36.10]) (Fig. 2B, Table 1).

Hemocytes were further analysed based on their ability to absorb SYTO62 and LysoTracker Green dyes. Cells with a high fluorescence level for both dyes were denoted as LThighS62high. Cells with a low fluorescence level were denoted as LTlowS62low. As a result, hyalinocytes and granulocytes separated into two groups of cells based on the intensity of accumulation of both dyes (Fig. 2C). When the parameters of the forward scatter and the side scatter and the intensity of dye absorption were also taken into account, four groups of hemocytes could be distinguished. Two of them were represented by hyalinocytes (FSlowSSlowLTlowS62low and FSlowSSlowLThighS62high), while the other two were represented by granulocytes (FShighSShighLTlowS62low and FShighSShighLThighS62high) (Table 1).

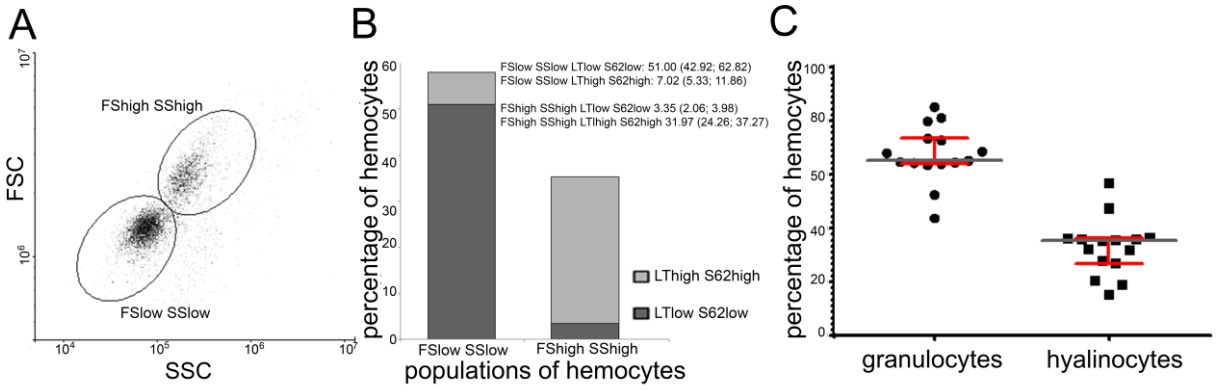


**Fig. 2** Population of hemocytes of *B. glabrata* (n=15) distinguished based on the cytometric assay: A – populations of hemocytes distinguished by forward scatter and side scatter: FShighSShigh – granulocytes, FSlow SSlow – hyalinocytes; B – relative number of granulocytes and hyalinocytes; C – the main populations of hemocytes identified based on size, granularity and absorption of SYTO62 and LysoTracker

**Table 1** Populations of cells in the hemolymph of the snail *B. glabrata*

| **Populations of hemocytes identified based on morphological study** | | | | |
|---|---|---|---|---|
| | Granulocytes | | Hyalinocytes | |
| Percentage | 73.3 ± 5.1 | | 23.7 ± 3.2 | |
| **Populations of hemocytes identified based on FS/SS** | | | | |
| | Granulocytes | | Hyalinocytes | |
| Percentage | 35.34 (27.38; 36.10) | | 65.13 (64.11; 73.05) | |
| Characteristics | FShighSShigh | | FSlowSSlow | |
| **Populations identified based on absorption of LysoTracker and SYTO62** | | | | |
| Percentage | 3.35 (2.06; 3.98) | 31.97 (24.26; 37.27) | 51.00 (42.92; 62.82) | 7.02 (5.33; 11.86) |
| Characteristics | LTlowS62low | LThighS62high | LTlowS62low | LThighS62high |

**Discussion**

The number of hemolymph cells is an important characteristic of the state of the internal environment of the mollusc. Its changes may be due to immunization resulting, e.g., from trematode infection (Ataev and Coustau, 1999; Ataev *et al.,* 2016).

Cell-counting chambers such as Goryaev's chamber or Neubauer´s chamber or multiwell glass slides have been traditionally used for counting hemocytes. It has been found with the use of these methods that 1 µl of hemolymph of *B. glabrata* contains ca. 120-140 hemocytes (Ataev and Coustau, 1999; Azevedo *et al.,* 2006; Pereira *et al.,* 2008 and others). In this study we obtained similar results: both manual counts and a more objective method of flow cytometry yielded the result of about 140 cells in 1 µl of hemolymph of *B. glabrata*.

The results of morphological studies of hemocytes agree with most reports about the main morphotypes of cells circulating in the hemolymph. Hyalinocytes differ from other cells in having higher NCR values. They do not contain any accumulations of granules and cannot properly spread on the substrate. On the other hand, granulocytes spread on the substrate and contain accumulations of granules (Abdul-Salam and Michelson, 1980; Barcante *et al*., 2012; Cavalcanti *et al*., 2012; Yoshino *et al*., 2013; Pila *et al*., 2016).

Granulocytes are more polymorphic as to size and granularity than hyalinocytes (Cheng, 1975; Joky *et al.,* 1983). Several researchers even distinguish two subpopulations of these cells. For instance, in a previous study we divided the granulocytes of planorbids *P. corneus*, *B. glabrata* and *P. planorbis* into two cell populations based in the results of a cytometric assay (Prokhorova *et al.,* 2018). Cells of these populations differed in the degree of granularity, the size and the ability to accumulate specific lysosomal and nucleic dyes (Prokhorova *et al.,* 2018). It cannot be ruled out, however, that these two groups of granulocytes belong to the same cell type and that the differences noted between them are due to functional specialization.

Flow cytometry data indicate that hyalinocytes are the prevailing cell type in the hemolymph of *B. glabrata.* They made up, on the average, 65% of all hemocytes. This agrees with the data on biomphalarians as well as other planorbids (Ataev *et al.,* 2016; Prokhorova *et al.,* 2018). However, counts of hemocytes on fixed preparations showed that granulocytes were more numerous (Table 1). Granulocytes also predominate on fixed preparations of other studied snails *such as P. corneus, P. planorbis* and *Pomacea canaliculata* (Cheng, 1975; Ottaviani, 1989; Azevedo *et al.,* 2006; Cueto *et al.,* 2015; Ataev *et al.,* 2016). This may be an artefact since hyalinocytes, which are less capable of adhesion, are lost in greater numbers than granulocytes during the preparation of fixed smears. At the same time, hemocyte concentration in 1 µl was similar regardless of whether it was determined by hemocytometer counts or by flow cytometry. This means that about 50% of hyalinocytes are lost during preparation of cells for microscopic observation owing to their lesser adhesive capacity. Therefore, flow cytometry is preferable for a quantitative analysis of the cell composition of the hemolymph because it does not incur any loss of cells.

Flow cytometry is a more objective method in this respect. Once the limits for the gating of cell population are set up properly, the proportion of circulating cells in numerous samples can be determined. However, this method needs to be adapted for each molluscan species.

Functional state of the cells can be assessed with the use of LysoTracker and SYTO62. Based on the accumulation of these specific dyes, four populations of hemocytes were distinguished in *B. glabrata*. Subpopulations of granulocytes and hyalinocytes were distinguished with the help of sequential gating based on the side scatter, the forward scatter and accumulation of the dyes. Most hyalinocytes accumulate both dyes in small amounts. They contain lysosomes and are not very active metabolically. On the contrary, most granulocytes are strongly granulated and metabolically active cells. Morphological data support this conclusion: accumulation of granules and rough reticulum in granulocytes and the dominance of euchromatin in their nuclei are well visible in electron micrographs.

Thus, the results of the flow-cytometric analysis confirmed the information about the composition of hemocytes in *B. glabrata* based on morphological data and provided a better understanding of their functional characteristics.

**Acknowledgements**

This investigation was financially supported by a grant of the Ministry of Education and Science of Russia (6.7509.2017/8.9) and a grant of the Russian Foundation for Basic Research (18-34-00522 mol_a).

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