

RESEARCH REPORT

Study of the proliferative activity of hemolymph cells in pulmonate molluscs**AS Tokmakova¹, MK Serebryakova², EE Prokhorova¹, GL Ataev^{1*}**¹Laboratory of Experimental zoology, Department of Zoology, Faculty of Biology, Herzen State Pedagogical University of Russia, St. Petersburg, Russia²Department of Immunology, Institute of Experimental Medicine, St. Petersburg, Russia*This is an open access article published under the CC BY license**Accepted May 7, 2020***Abstract**

Hemocytes, the cell of the hemolymph, play a key role in the immune response of pulmonate molluscs to various pathogens including trematode infection. The number of hemocytes is known to increase after immunization but the mechanism of their multiplication remains debatable, proliferative capacity being the stumbling block. Some scientists consider that hemocytes may proliferate, while others think that their multiplication is only possible in special hematopoietic centres. In this work we studied the proliferative activity of hemocytes in five species of pulmonate molluscs: *Biomphalaria glabrata*, *Planorbis corneus*, *Planorbis planorbis*, *Lymnaea stagnalis* and *Succinea putris*. ImageStream technique was used for the study of the hemocyte populations of these molluscan species for the first time. The hemocytes of all the studied species were represented by two main types, granular cells and hyalinocytes. Microscopic and flow-cytometric study of the hemocytes with the use of EdU revealed some EdU-positive cells. However, the analysis of the cell cycle of the hemocytes showed that the amount of DNA in these cells was not increased. Thus, it remains unclear whether the cells of the hemolymph retain the capacity to multiplication.

Key Words: pulmonate molluscs; hemocytes; proliferation; EdU; flow cytometry; ImageStream**Introduction**

Hemolymph cells of pulmonate molluscs, hemocytes, are usually considered as the major effectors of molluscan defence reactions to various pathogens including trematode invasion (Sminia *et al.*, 1974; Lie and Heyneman, 1976; Van der Knaap and Loker, 1990; Ataev and Coustau, 1999; Connors, 2003; Pila *et al.*, 2016). Hemocytes are generally divided into two main types: granular cells (the term granulocytes is also widely used to refer to these cells) and hyalinocytes (see Discussion).

Despite intensive studies of morphology and functional activity of hemocytes, there is still no consensus about the nature and the mechanism of hemopoiesis in pulmonate molluscs. Many authors consider that the amoebocyte-producing organ (APO), located between the pericardial and the mantle epithelium, is a common hemopoietic centre (Pan, 1958; Jeong *et al.*, 1983; Joky *et al.*, 1983; Sullivan, 1988). Immunization of molluscs with various pathogens is shown to intensify the

proliferation of its cells (Lie and Heyneman, 1976; Sullivan, 1988; Ataev and Prokhorova, 2013). Other scientists think that the origin of hemocytes is polycentric and that they may also form from the cells of the molluscan connective tissue (Souza and Andrade, 2006). According to a third hypothesis, the proliferation of circulating cells of the snail hemolymph is also possible (Sminia *et al.*, 1983; Monteil and Matricon-Gondran, 1991; Portet *et al.*, 2019).

The proliferative potential of cells is often assessed with the use of BrdU (Dolbeare, 1995; Terry and White, 2001; Rodriguez *et al.*, 2018 *et al.*) but this technique has limitations. One of them is that DNA denaturation, which is necessary for revealing the label, also destroys most protein epitopes. These complications can be avoided by using a technique based on 5-ethynyl-2-deoxyuridine (EdU) (Buck *et al.*, 2008; Cappella *et al.*, 2008). Being an analogue of thymidine, EdU allows one to register the instances of DNA synthesis in the nuclei. This technique has mostly been used for the study of cells of vertebrates (Salic and Mitchison, 2008; Lin *et al.*, 2009; Chehrehasa *et al.*, 2009; Zeng *et al.*, 2010; Fabrice *et al.*, 2015) but Portet *et al.* (2019) successfully used it to determine proliferative activity of hemolymph cells in molluscs

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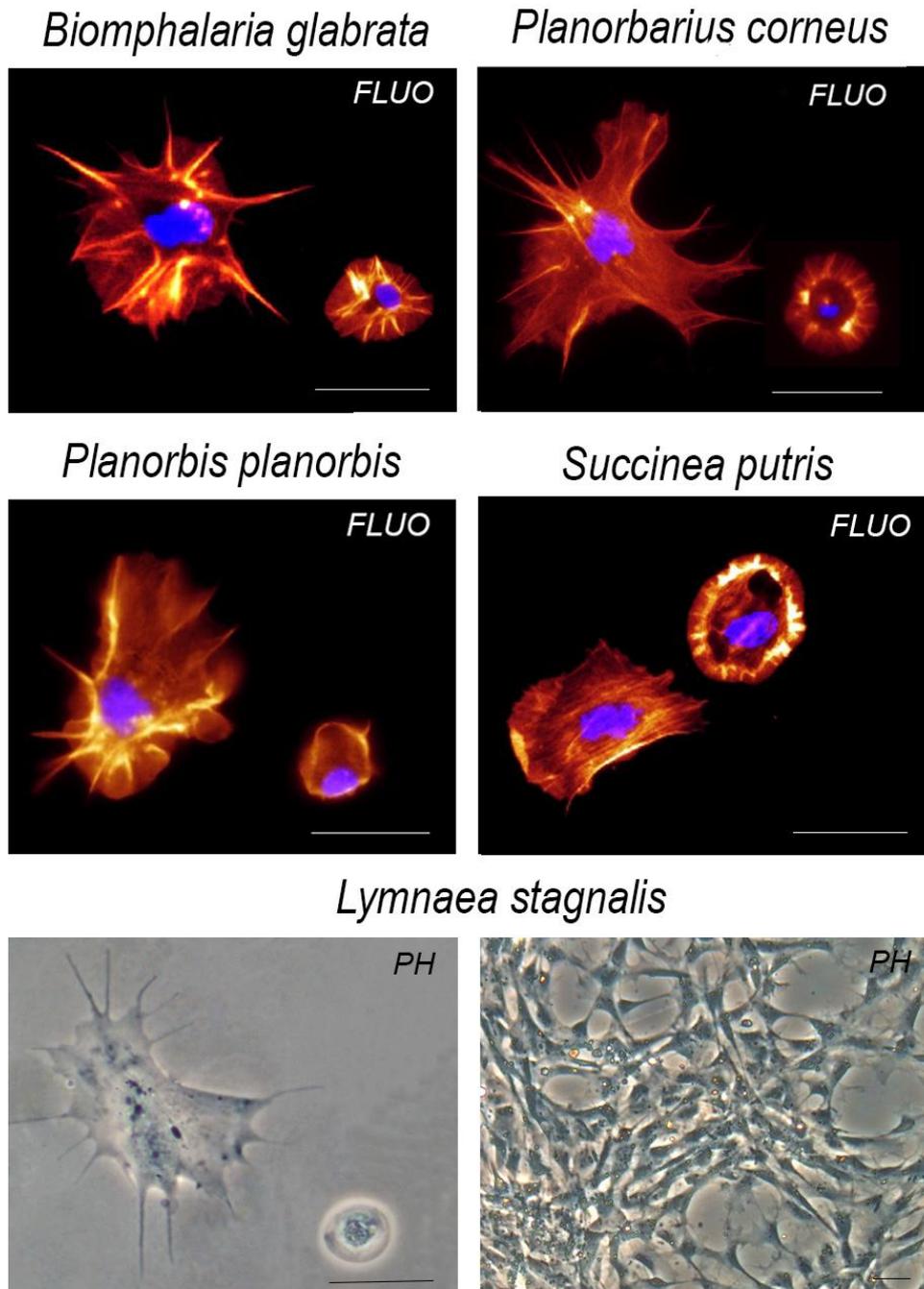


Fig. 1 Hemocyte morphology in pulmonate molluscs *B. glabrata*, *P. corneus*, *P. planorbis*, *S. putris*, *L. stagnalis*. FLUO – fluorescence images of phalloidin-rhodamin (red) and Hoechst (blue) stained hemocytes, PH – Phase contrast microscopy images of living cells. Bar = 10 μ m

Biomphalaria glabrata. They showed that EdU accumulated in the nuclei of some hemocytes and interpreted this observation as an evidence of the possible proliferation of the circulating cells (Portet *et al.* 2019). However, the accumulation of EdU may also indicate some other processes such as reparation.

We agree with the hypothesis that hemocytes of pulmonates multiply only in special centres such as APO. These centres probably contain stem

hematopoietic cells, which can divide and differentiate into the hemolymph cells. At the same time, there are occasional reports about the proliferative capacity of the circulating cells of gastropods, including pulmonates. To check this capacity, we conducted a complex study with the use of cytometric and microscopic methods of five species of pulmonate molluscs: freshwater snails *B. glabrata*, *Planorbarius corneus*, *Planorbis planorbis*, and *Lymnaea stagnalis* and a land snail *Succinea putris*.

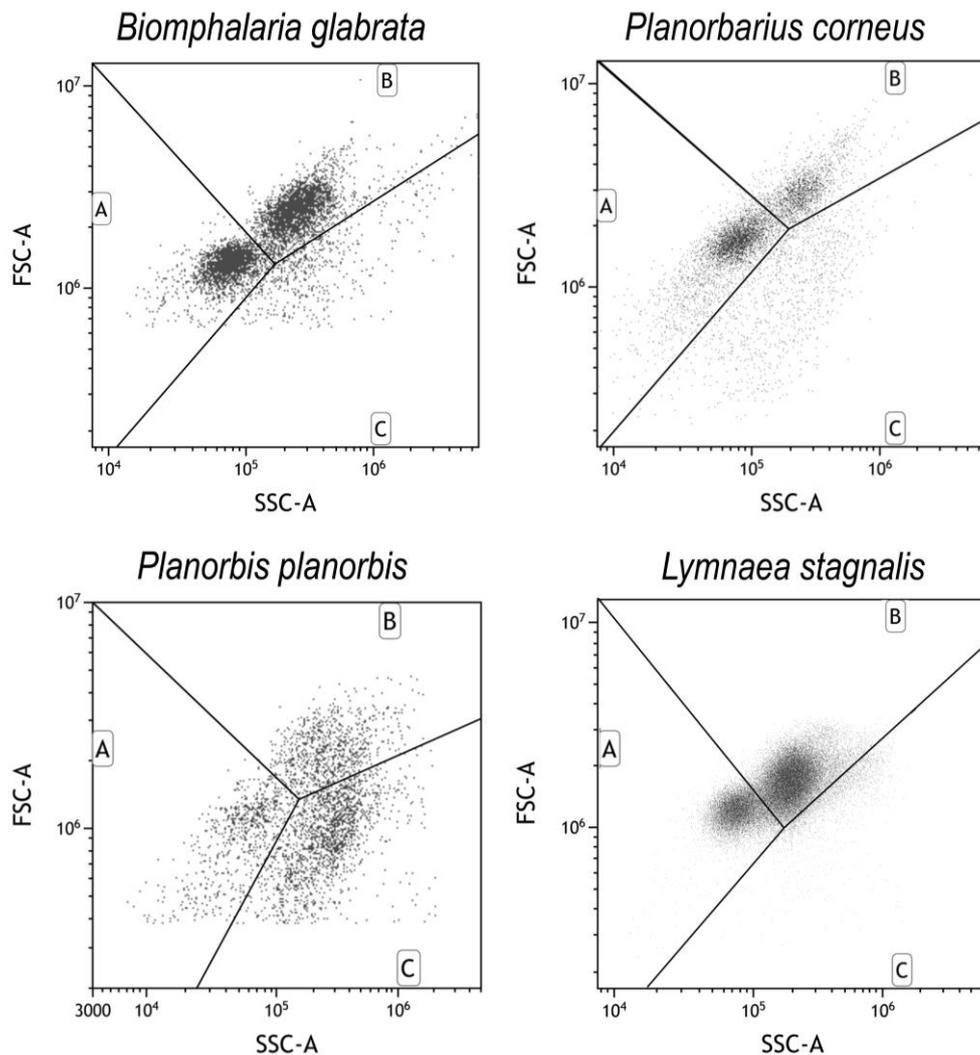


Fig. 2 Hemocyte populations of molluscs *B. glabrata*, *P. corneus*, *P. planorbis* and *L. stagnalis* identified by flow cytometry based on forward (FSC) and side (SSC) scatter. Population A corresponds to hyalinocytes, while populations B and C are subpopulations of granular cells

Materials and methods

Snails

Freshwater snails *Planorbarius corneus* L., 1758, *Planorbis planorbis* L., 1758, *Lymnaea stagnalis* L., 1758 and land snails *Succinea putris* L., 1758 used in this study were collected in the Leningrad Region of Russia. Snails *Biomphalaria glabrata* Say, 1818 (laboratory strain) were obtained from the “Interactions Hôtes-Pathogènes-Environnements” Laboratory of the Perpignan University (France) in 2011. The strains of *P. corneus* have been kept in the laboratory since 2010 (Prokhorova *et al.*, 2015). The snails of all species were kept in aerated water tanks on a 12L/12D cycle at a temperature of 22 - 23 °C.

The snails collected in natural water bodies were brought to the laboratory and placed into glass jars. The jars were screened for the presence of cercariae, which are shed by snails infected with

trematodes. The hemolymph of the seemingly uninfected snails was sampled, and the snails were dissected to ascertain the lack of infection. Only the hemolymph of uninfected snails was used for the study. All the hemolymph samples were analysed immediately after sampling. Land snails *S. putris* (Prokhorova *et al.*, 2020). were kept in containers with moist soil (temperature 22 - 23 °C, air humidity 60 - 70 %). Snails collected in the nature were kept in the laboratory for not more than 1 - 2 before the experiment. All snails were fed on lettuce leaves once in two days.

Hemolymph collection, incubation and sample preparation

Hemolymph was collected from the pericardial region of the snail using glass Pasteur pipettes (Sminia, Baredsen, 1980). Morphological analysis of hemocytes was performed on temporary and fixed preparations. For *in vitro* studies, freshly sampled

Table 1 Relative number of cells of different populations in the hemolymph of molluscs *B. glabrata*, *P. corneus*, *P. planorbis*, *L. stagnalis* based on cytofluorimetric analysis.

Snail species (sample size)	Hemocyte populations based on forward and side scatter		
	A	B	C
<i>Biomphalaria glabrata</i> (n = 20)	54.09 (45.97; 63.68)*, **	28.80 (20.67; 32.03)*, ^	13.98 (11.58; 18.59)*, ^
<i>Planorbarius corneus</i> (n = 18)	44.14 (37.15; 55.26)*	35.66 (26.13; 41.36)*, ^	18.49 (13.10; 26.84)*, ^
<i>Planorbis planorbis</i> (n = 19)	48.03 (38.58; 59.59)*	16.11 (12.07; 23.30)*	28.39 (20.14; 38.62)*
<i>Lymnaea stagnalis</i> (n = 22)	11.56 (8.06; 20.74)	83.03 (72.01; 87.46)	4.66 (3.59; 7.35)

Data are given as percentage from the total number of analysed single cells in the sample as median values and interquartile range (% , Me [Q25; Q75]). * - $p < 0.001$ when compared with *Lymnaea stagnalis*, ** - $p < 0.05$ when compared with *Planorbarius corneus*, ^ - $p < 0.05$ when compared with *Planorbis planorbis*, ^^ - $p < 0.005$ when compared with *Planorbis planorbis*

hemolymph was smeared on object slides with an adhesive covering and analysed using phase contrast. For phalloidin staining, smears of hemocytes were fixed in 4 % paraformaldehyde in PBS and incubated for 10 min. After washing twice in PBS, cells were preincubated in PBS with 0.1 % TritonX-100 for 5 min and then incubated with Phalloidin-Rhodamin (Thermo Fisher Scientific) diluted with PBS for 30 min at room temperature. The nuclei of hemocytes were counterstained with Hoechst (ThermoFisher Scientific).

To study proliferative activity of hemocytes, the hemolymph was incubated with 20 mM EdU for 30 min on object slides in a moist chamber. EdU label was exposed with the use of Click-iT EdU Alexa Fluor 594 Imaging Kit according to the manufacturer's protocol (ThermoFisher Scientific). The nuclei of hemocytes were counterstained with Hoechst (ThermoFisher Scientific).

The preparations were analysed using a fluorescent microscope Leica DMI8. The images were processed with the help of LASX software.

Flow-cytometric assay

The composition of the population of hemolymph was studied using flow cytometer BD Accuri C6 (BD Bioscience, USA). For this, freshly drawn hemolymph was diluted with Chernin buffer (Chernin, 1968) in the proportion of 1 : 1 (for *P. corneus* and *B. glabrata*) and 1 : 4 (for *P. planorbis*). Hemolymph of *L. stagnalis* was studied without dilution. Hemolymph samples were not pooled, and the hemolymph of each individual was analysed separately. The composition of the hemocyte population was determined based on forward and side scatter (see: Prokhorova *et al.*, 2018a, b). The debris was removed based on cell staining with a vital stain SYTO62 (Invitrogen, USA) specific to nucleic acids. The stain was added to the freshly sampled hemolymph (final concentration, 250 nM), incubated for 10 min and immediately analysed using the flow cytometer. SYTO62-negative events were considered as debris and excluded from the analysis zone. The aggregates were excluded successively based on the proportion of the peak

(H) and the integral signals (A) registered for the parameters of the forward and the side scatter. At least 5,000 events were analysed for each hemolymph sample of *B. glabrata*; at least 20,000 events for *P. corneus*, at least 30,000 events for *L. stagnalis*.

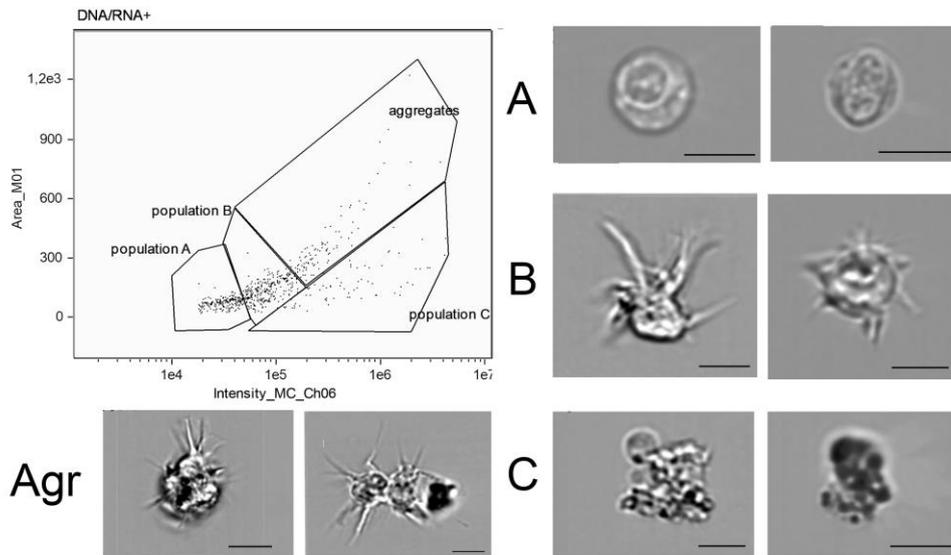
For the analysis of proliferative activity of hemocytes with the help of flow cytometry, the hemolymph was incubated with 20 mM EdU for 30 min in cytometric tubes (Sarstedt, Germany). EdU was exposed as described above. At the last stage of the sample preparation, DAPI DNA-binding stain was added to the samples for the subsequent analysis of the distribution of the circulating hemolymph cells across the cell cycle stages (Firsanov *et al.*, 2017). Monocytic leukemia THP-1 cells, for which the amount of proliferating cells in the culture is about 30 %, were used as positive control. Samples for THP-1 were prepared according to the protocol used for the preparation of hemocytes. Hemocytes not incubated with EDU but subjected to all the previous stages of the protocol were used as negative control. The samples were analysed with the use of a Navios 10/3 flow cytometer (Beckman Coulter, USA). Not less than 5000 DNA-containing events were analysed for each sample.

ImageStream assay

We also used an Amnis® ImageStream® imaging flow cytometer (Luminex, USA), which makes it possible to obtain cytometric data about a hemolymph sample and simultaneously to visualize events on a cytogram.

Signals characterising cell morphology (Ch01, Ch06) and fluorescence SYTO62 by Ch11 channel were registered. Not less than 200,000 events, including gauge particles, were analysed for all the samples. The data obtained with the help of ImageStream technology were used to construct a two-parameter histogram, where the values of cellular complexity (Intencity MC Ch06) were plotted along the x-coordinate and the values of cell area (Area M01) were plotted along the y-coordinate. Area M01 is the parameter closest to the forward scatter of the classical flow cytometer.

Biomphalaria glabrata



Planorbarius corneus

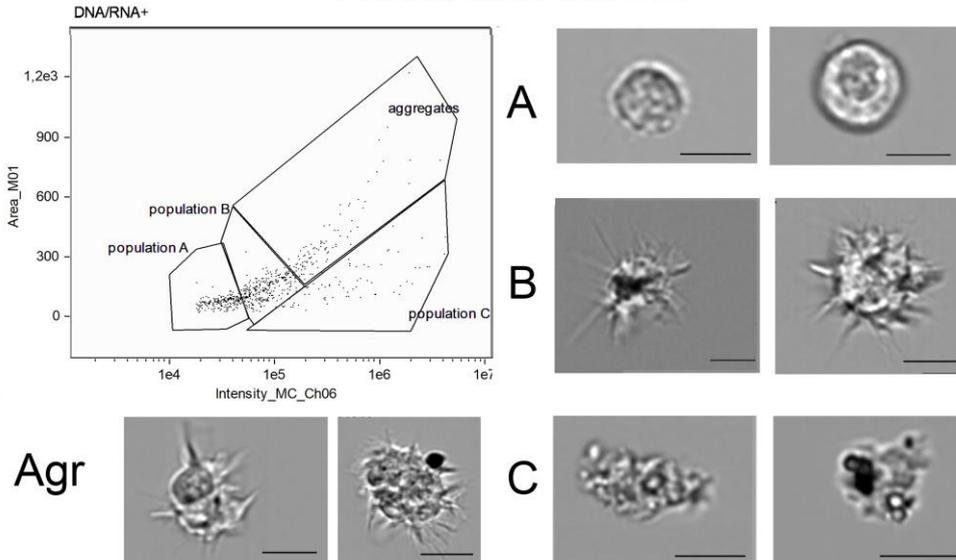


Fig. 3 Populational analysis of circulating hemocytes of molluscs *B. glabrata* and *P. corneus* performed with the help of Amnis® ImageStream imaging flow cytometer. Hemolymph histograms (x-coordinate – cellular complexity, y-coordinate – cell area) and visualised events are shown. A, B, C — hemocyte populations based on forward and side scatter, Agr — aggregates. Bar = 7 μ m

Statistical analyses

The cytometric data were processed using Kaluza™ v.2.0 software (Beckman Coulter, USA). Cell cycle parameters were calculated in the automatic mode with the help of CellCycle module incorporated in Kaluza™ v.2.0. Statistical treatment was conducted with the use of MS Excel (MicroSoft, USA), Statistica 8.0 (StatSoft, USA) and GraphPad Prism 6.01 (GraphPad Software Inc, USA). The normality of distribution in the samples was assessed with the help of Kolmogorov-Smirnov's test. The results were represented as a median (Me) and an interquartile range (Q25, Q75).

Results

Morphological types of hemocytes

In micrographs obtained with the help of light and fluorescent microscopes, the hemocytes of all studied molluscs could be divided into two types: granular cells and hyalinocytes (Ataev *et al.*, 2016; Prokhorova *et al.*, 2018 a,b). All hemocytes have a well-developed actin cytoskeleton ensuring shape maintenance (Fig. 1). Granular cells are larger, contain numerous granules, form filopodia and spread out easily on the substrate. Hyalinocytes are agranular, rounded and may form lobopodia.

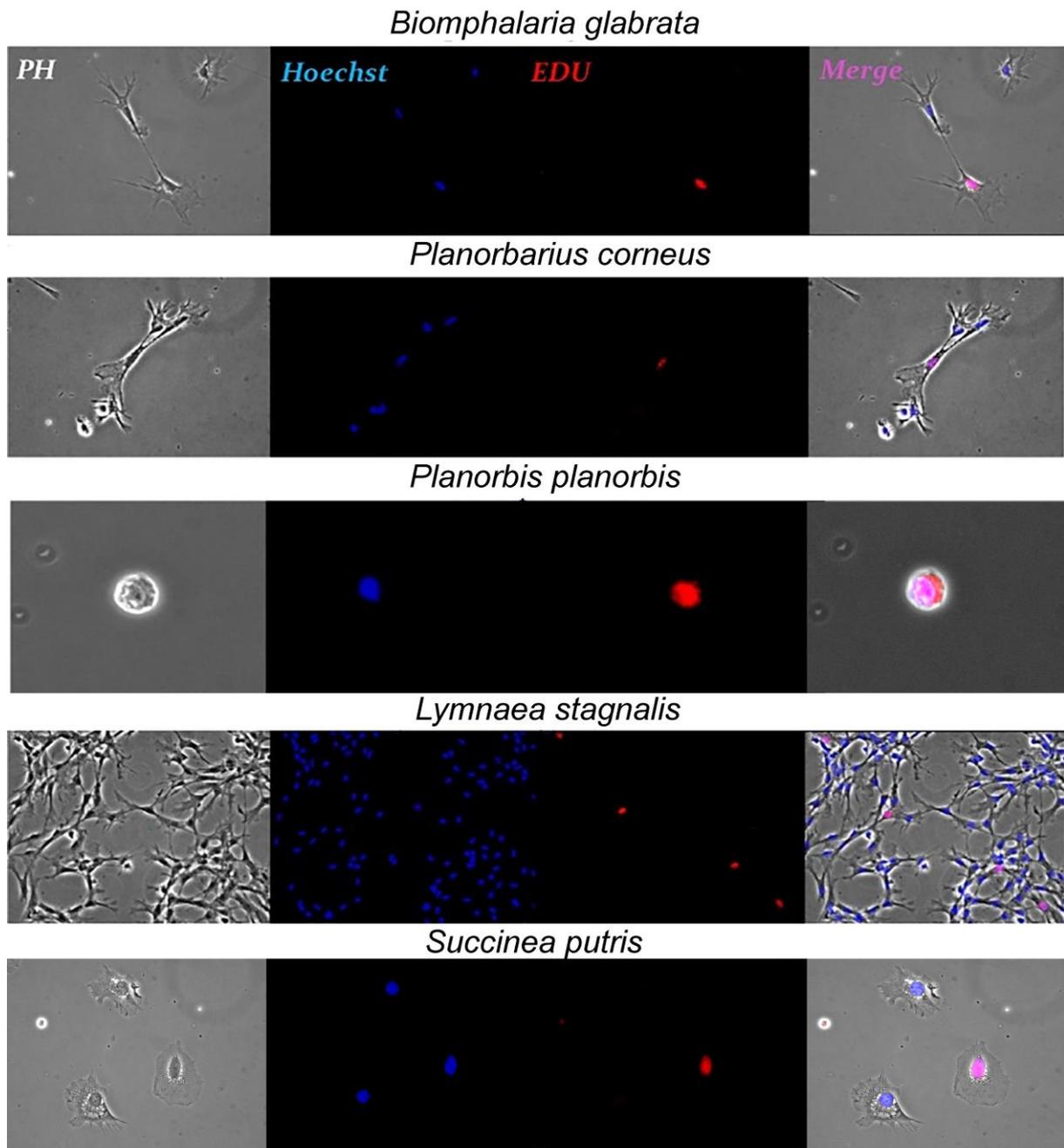


Fig. 4 EdU incorporation in nuclei of hemocytes of pulmonate molluscs. PH -phase-contrast microscopy. FLUO – fluorescence photomicrographs of hemocyte nuclei stained with Hoechst (blue) and EdU (red)

Pools of hemocytes detected by flow cytometry

Based on the results of the cytofluorimetric analysis of the hemolymph, the hemocytes were divided into two main populations: A and B (Fig. 2, Table 1). The values of forward and side scatter indicated that the cells of population A were small and had a relatively simple structure. The cells of population B were located further along both x- and y-coordinate, that is, were larger and more complexly organised because of the presence of numerous granules and vesicles. Depending on the snail

species, the hemolymph contained from 5 to 29 % of heterogeneous hemocytes, which were, on the average, somewhat smaller than granular cells of population B (Table 1). Based on the results of the cytometric analysis, we denoted these as a separate subpopulation C (Fig. 2). This group of cells was especially noticeable in *P. planorbis* (Table 1).

In general, the proportion of different cell populations in the hemolymph varied in molluscs of different species. For instance, more than 80 % of the

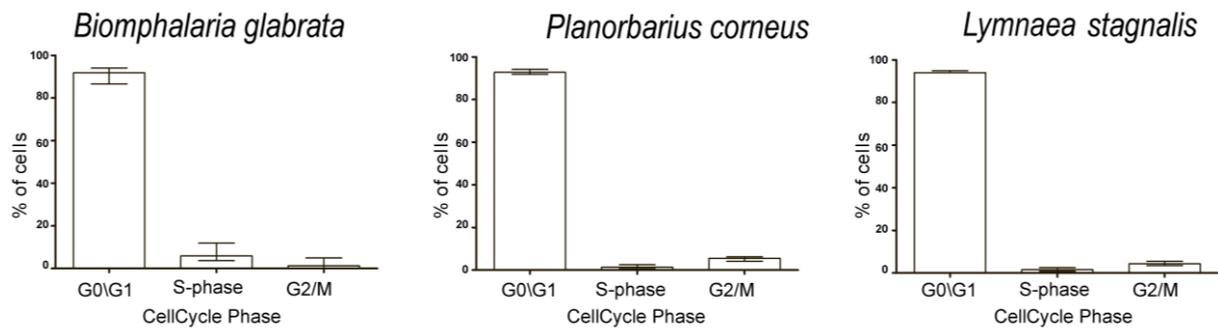


Fig. 5 Distribution of molluscan hemocytes across cell cycle phases based on cytofluorimetric analysis of DAPI-treated hemolymph

hemocytes of *L. stagnalis* were granular cells, which means this snail differs statistically significantly ($p < 0.0001$) from the other snail species, in which hyalinocytes predominated.

The hemolymph of molluscs *B. glabrata* ($n = 10$) and *P. corneus* ($n = 10$) was also analysed with the help of ImageStream technique (Fig. 3). The results confirmed the presence in the hemolymph of two main cell types, hyalinocytes and granular cells (population A and B). However, it can be seen on the cytograms that subpopulation C includes small strongly vacuolated cells that look like cells at the terminal stages of the apoptosis, as well as cell debris. In this way, the results of ImageStream analysis made it possible to correct the gating tactics during the analysis of the cytograms obtained with the use of flow cytometer and to exclude the cells of population C from the analysis.

Proliferative activity of the hemocytes and cell cycle

To study the proliferative activity of the hemocytes, we conducted microscopic (Fig. 4) and cytometric analysis (Fig. 5-8) of EdU incorporation into DNA of molluscan hemocytes at the background of their distribution across the cell cycle phases. We found that EdU accumulated in the nuclei of some hemocytes of molluscs *B. glabrata* ($n = 42$), *P. corneus* ($n = 45$), *P. planorbis* ($n = 10$), *L. stagnalis* ($n = 29$) and *S. putris* ($n = 12$). These results in general confirmed the data obtained on *B. glabrata* by Portet *et al.* (2019).

At the same time, the analysis of the cell cycle showed that most of the hemocytes were in the phase G0/G1 (Fig. 5). The percentage of hemocytes in this phase from the total number of these cells made up 91.82 (86.60; 94.01) % for *B. glabrata*, 92.77 (92.00; 93.94) % for *P. corneus* and 93.98 (92.92; 94.88) % for *L. stagnalis*. The percentage of hemocytes in the synthesis phase was 5.92 (4.93; 9.08), 1.37 (0.79; 2.45) and 1.55 (0.93; 2.51) % for *B. glabrata*, *P. corneus* and *L. stagnalis*, correspondingly. DNA amount of other hemocytes corresponded to phase G2 and mitosis. The number of such cells was 1.31 (0.31; 4.12), 5.51 (4.33; 6.19) and 4.36 (3.65; 5.44) % for *B. glabrata*, *P. corneus* and *L. stagnalis*, correspondingly.

Cytometric analysis of EdU incorporation into the DNA of hemocytes showed that most of the EdU-positive cells (Fig. 6a) were located in the upper right part of the FS/SS histogram (Fig. 6b). However, an important aspect of cytometric study of proliferative activity of any cells is the exclusion of aggregates from the analysis. We identified single cells based on the proportion of the peak and the integral signal of DAPI fluorescence and showed that most of the EdU-positive events were situated in the area of aggregates and cell debris containing a lowered amount of DNA (Fig. 6c). Monocytic leukemia THP-1 cells, for which the amount of proliferating cells in the culture is about 30 %, were used as positive control (Fig. 7).

After the exclusion of cell aggregates and cell debris from the analysis zone, EdU-positive hemocytes in *B. glabrata* made up 5.8 % (5.57; 6.21) of the total number of single cells (Fig. 6c). Most of these cells, 4.02 % (3.45; 4.94), were located in the area of G0/G1 peak based on the amount of DNA (Fig. 8). In *P. corneus* the percentage of EdU-positive hemolymph cells was lower, making up 2.89 % (1.59; 3.53) of the total number of single hemocytes. More than a half of them, 2.03 % (0.78; 2.72), were located in the area of G0/G1 peak (Fig. 8). However, for *L. stagnalis* the number of stained cells in the samples incubated with EdU did not exceed the corresponding value for negative control, and so no conclusions about EdU incorporation into the DNA of the hemocytes could be made (Fig. 7).

Discussion

Classification of hemocytes in pulmonates remains debatable. From one to several tens of types of hemolymph cells have been described. However, most authors (Sminia, 1972; Cheng, 1975; Jourdane and Cheng, 1987; Ataev *et al.*, 2016; Pila *et al.*, 2016) identify two main populations of hemocytes based on morphological characters: hyalinocytes (agranular cells) and granular cells (easily spreading cells, whose cytoplasm contains numerous granules and vesicles). The difference between hemocyte types has been shown both at the light and at the electron-microscopic level (Joky *et al.*, 1983; Ottaviani and Franchini, 1988; Cueto *et*

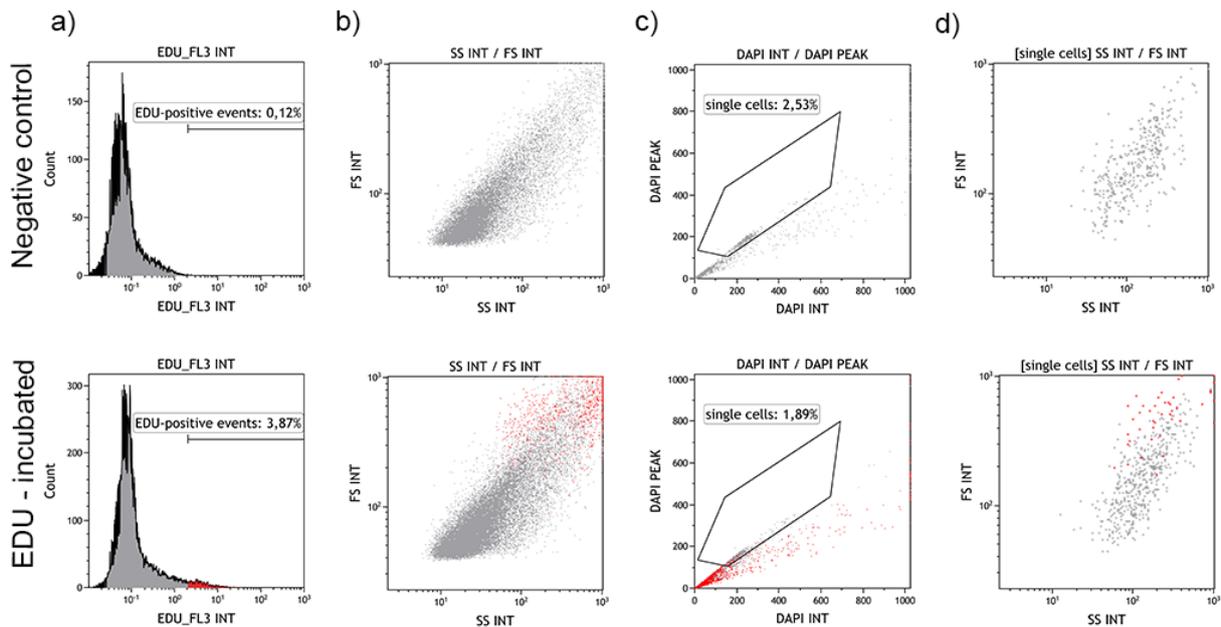


Fig. 6 Cytofluorimetric analysis of EdU incorporation into hemocytes of molluscs *B. glabrata* a) Identification of EdU-positive events from all events in a sample. x-coordinate –EdU fluorescence intensity, y-coordinate – number of cells b) Localization of EdU-positive events (red) on the histogram, where SS axis is side scatter and FS axis is forward scatter. All registered events are presented on the histogram c) Identification of single cells based on DAPI incorporation, exclusion of aggregates and debris. DAPI INT axis — integral signal of DAPI fluorescence intensity, DAPI PEAK axis — peak signal of DAPI fluorescence intensity. EdU-positive events are shown in red d) Localization of EdU-positive events (red), where SS axis is side scatter and FS axis is forward scatter. Only single cells are shown in the histogram

al., 2015; Prokhorova *et al.*, 2018b and others). However, morphological characters of hemocytes of different types have never been clearly formulated because of their polymorphism. This might be the reason why additional types (subpopulations) of hemolymph cells of snails have often been described (Joky *et al.*, 1983; Cavalcanti *et al.*, 2012; Cueto *et al.*, 2015; Rodriguez *et al.*, 2018). In our

earlier studies we also described four subpopulations of cells with different functional characteristics in each hemocyte population in the hemolymph of *B. glabrata*, *P. corneus* and *P. planorbis* based on cytometric analysis with the use of specific fluorescent dyes, a lysosome dye (LysoTracker) and a nucleic acid dye (SYTO62) (Prokhorova *et al.*, 2018a,b).

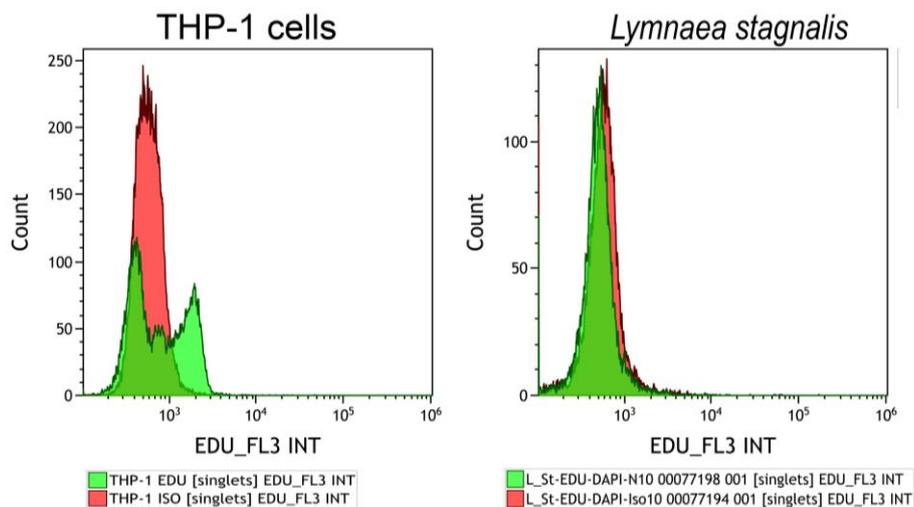


Fig. 7 Histograms showing superimposition of fluorescence signals of cells incubated with EdU (green) and negative control – cells without incubation with EdU (red)

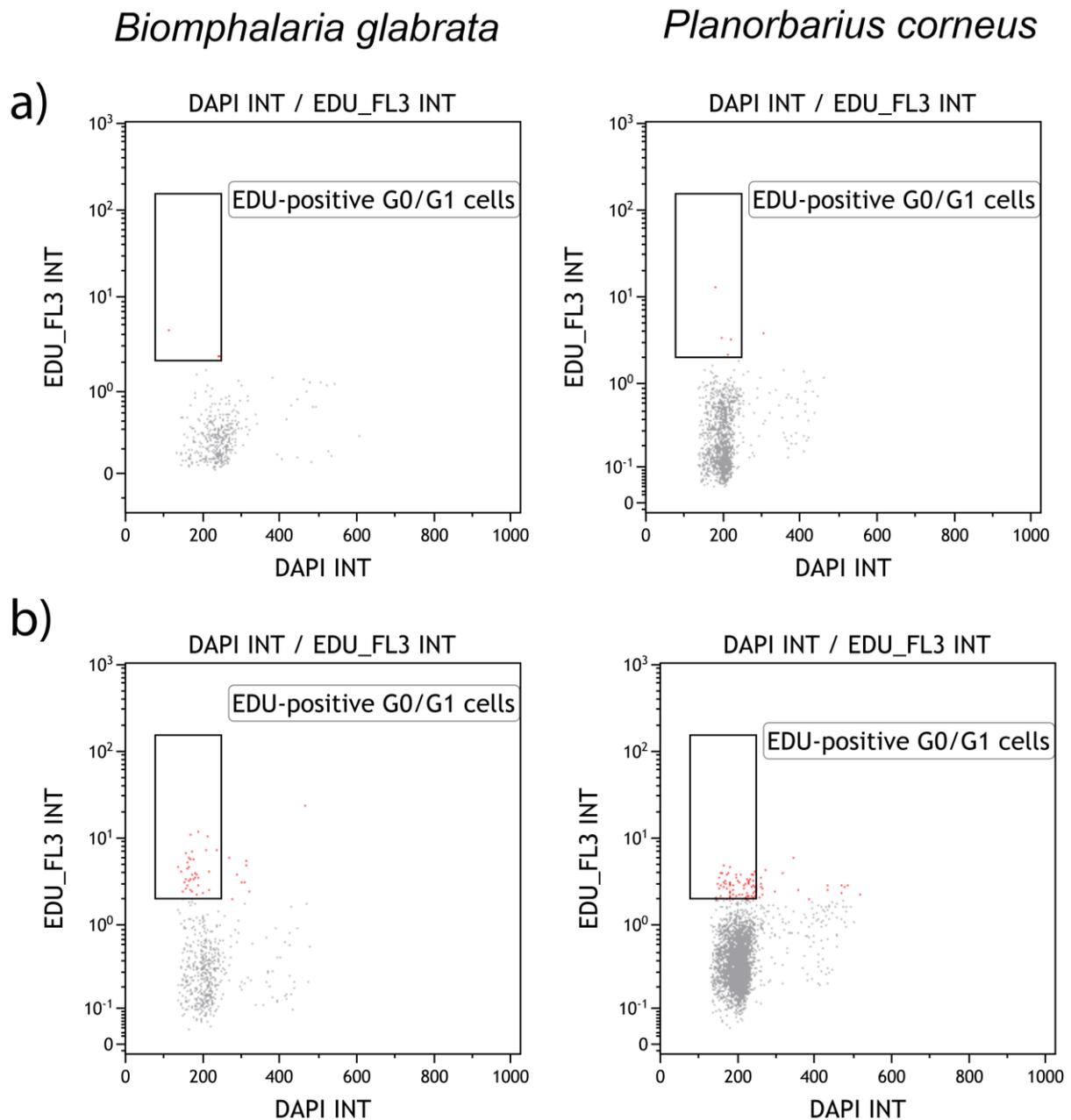


Fig. 8 Cytofluorimetric analysis of hemocyte distribution across cell cycle phases with the use of DAPI and EdU. a) Negative control without EdU incubation, b) Experiment. DAPI INT axis — integral signal of DAPI fluorescence intensity, EdU_FL3_INT axis — integral signal of EdU fluorescence intensity. EdU-positive events corresponding to G0/G1 phase are shown in red

The results of this study confirmed that the circulating cells in of hemolymph of *B. glabrata*, *P. corneus*, *P. planorbis*, *L. stagnalis* and *S. putris* were represented by two main populations: granular cells and hyalinocytes. These cells differ in their morphology, adhesive capacity and functional activity (Ataev *et al.*, 2016; Prokhorova *et al.*, 2018 a,b).

As noted above, the classification of hemocytes based on morphological characters may not fully reflect the functional features of these cells, which

may be expressed during their specialization. For instance, at first, with the help of flow cytometric analysis of the hemolymph of *B. glabrata*, *P. corneus*, *P. planorbis* and *L. stagnalis*, we revealed subpopulation C represented by small granular cells (Fig. 2). However, the use of ImageStream technology showed that this population was represented by degranulating cells and/or cells undergoing apoptosis. As a result, we excluded these cells from the further analysis.

Thus, the use of ImageStream technique in general confirmed the results obtained by flow cytometry. Based on the size and signal intensity assessing the cellular complexity, hemocytes were represented by two main populations (Fig. 3). They evidently corresponded to population A (hyalinocytes) and population B (granular cells) identified based on morphological and cytometric analysis (Fig. 1, 2).

The study of the hemolymph of *B. glabrata* with the use of EdU confirmed the results of Portet *et al.* (2019) that EdU is incorporated into hemocyte nuclei. These authors have reported the relative number of these cells based on the results of microscopic analysis: 1.16 % in uninfected snails; 2.57 % in snails with sympatric infection; 5.19 % in snails with allopatric infections. These results are difficult to comment upon since some hemocytes are lost in the process of making the preparations (especially during washing). However, the authors also report the results of flow cytometry of the hemolymph with the use of EdU. Their data generally show that the number of EdU-positive cells increased up to 2 % in molluscs with sympatric infection and up to 4.2 - 6.8 % in molluscs with allopatric infection.

We cannot provide a reliable percentage of EdU-positive cells based on microscopic analysis because such counts may be biased (see above). Based on cytometric analysis, the proportion of such cells in uninfected *B. glabrata* and *P. corneus* varies but also does not exceed several per cent. Moreover, the study of hemolymph of these molluscs with a simultaneous use of EdU and DAPI also revealed a small percentage of cells in phase S and phase G2/M. However, it is the EdU-positive cells that were found among hemocytes in phase G0/G1 (Fig. 8).

The incorporation of EdU into the nuclei of the circulating cells may be associated with the DNA repair in them. To the best of our knowledge, no studies of this kind have been conducted on molluscan hemocytes but there are such data for vertebrates (Kim *et al.*, 2019; Wiley *et al.*, 2019). In this case, the number of EdU-positive hemocytes may increase after immunization and, in particular, after infection of molluscs with trematodes.

Another explanation of this phenomenon is also possible. In vertebrates, bone marrow is the central organ of hemopoiesis but differentiated and extremely specialised cells, the macrophages, can proliferate in tissues (Lin and Stewart, 1973; Luo *et al.*, 2008; Robbins *et al.*, 2013). This mechanism, alongside with the recruitment of monocytes, is considered as an alternative means of increasing the numbers of tissue macrophages in damaged tissues during inflammation (Mlcochova *et al.*, 2017). Besides, clonal expansion of lymphocytes occurs in vertebrates during antigen-dependent differentiation (Fox and Smith, 1986; Janeway *et al.*, 2001; Zink *et al.*, 2017).

A similar mechanism may also be at work in pulmonates. In such a case, prohemocytes that have left the hemopoietic centre (APO) before differentiation into hemocytes may retain the ability to divide. In our opinion, it is also possible that a small percentage of hemocytes retain proliferative

capacity that may be activated under certain conditions. For instance, local proliferation of snail hemocytes may be stimulated by cytokines secreted by the cells of damaged tissues as well as metabolic products of pathogens.

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References

- Ataev GL, Coustau C. Cellular response to *Echinostoma caproni* infection in *Biomphalaria glabrata* strains selected for susceptibility/resistance. *Dev Comp Immunol.* 23: 187-198, 1999.
- Ataev GL, Prokhorova EE. Changes of the amebocyte-producing organ in *Biomphalaria glabrata* mollusks infested by *Echinostoma caproni* trematodes. *Parazitologiya.* 47: 472-479, 2013.
- Ataev GL, Prokhorova EE, Kudryavtsev IV, Polevshchikov AV. The influence of trematode infection on the hemocyte composition in *Planorbium corneus* (Gastropoda, Pulmonata). *ISJ.* 13: 164-171, 2016.
- Buck SB, Bradford J, Gee KR, Agnew BJ, Clarke ST, Salic A. Detection of S-phase cell cycle progression using 5-ethynyl-2'-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2'-deoxyuridine antibodies. *Biotechniques.* 44: 927-929, 2008.
- Cappella P, Gasparri F, Pulici M, Moll J. A novel method based on click chemistry, which overcomes limitations of cell cycle analysis by classical determination of BrdU incorporation, allowing multiplex antibody staining. *Cytometry A.* 73: 626-636, 2008.
- Cavalcanti MGS, Filho FC, Mendonc AMB, Duarte GR, Barbosa CCGS, De Castro CMMB *et al.* Morphological characterization of hemocytes from *Biomphalaria glabrata* and *Biomphalaria straminea*. *Micron.* 43: 285-291, 2012.
- Chehrehasa F, Meedeniya AC, Dwyer P, Abrahamsen G, Mackay-Sim A. EdU, a new thymidine analogue for labelling proliferating cells in the nervous system. *J Neurosci Methods.* 177: 122-130, 2009.
- Cheng TC. Functional morphology and biochemistry of molluscan phagocytes. *Ann N Y Acad Sci.* 266: 343-379, 1975.
- Chernin E. Observations on hearts explanted in vitro from the snail *Australorbis glabratus*. *Journ Parasitol.* 49: 353-364, 1968.

- Connors VA. The schistosome-snail interaction: factors involved in host immunodefense activation and parasite killing in susceptible and resistant *Biomphalaria glabrata*. *Taxonomy, ecology and evolution of metazoan parasites*. 1: 203-224, 2003.
- Cueto JA, Rodriguez C, Vega IA, Alfredo CV. Immune defenses of the invasive apple snail *Pomacea canaliculata* (Caenogastropoda, Ampullariidae): phagocytic hemocytes in the circulation and the kidney. *PLoS One*. 10: e0123964, 2015.
- Dolbear F. Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part I: Historical perspectives, histochemical methods and cell kinetics. *Histochem. J*. 27: 339-369, 1995.
- Fabrice A, Benoît R, Valérie N, Lau E, Sébastien B, Frédéric P. A simple method to assess in vivo proliferation in lung vasculature with EdU: the case of MMC-induced PVOD in rat. *Anal Cell Pathol (Amst)*. 2015: 1-6, 2015.
- Firsanov D, Solovjeva L, Lublinskaya O, Zenin V, Kudryavtsev I, Serebryakova M *et al*. Rapid detection of γ -H2AX by flow cytometry in cultured mammalian cells. *Methods Mol Biol*. 1644: 129-138, 2017.
- Fox DA, Smith BR. Evidence for oligoclonal B cell expansion in the peripheral blood of patients with rheumatoid arthritis. *Ann Rheum Dis*. 45: 991-995, 1986.
- Janeway CA, Travers P, Walport M, Shlomchik MJ. *Immunobiology: The Immune System in Health and Disease*. 5th edition. Garland Science, New York, 2001.
- Jeong KH, Lie KJ, Heyneman D. The ultrastructure of the amoebocyte-producing organ in *Biomphalaria glabrata*. *Dev Comp Immunol*. 7: 217-228, 1983.
- Joky A, Matricon-Gondran M, Benex J. Fine structural differences in the amoebocytes of *Biomphalaria glabrata*. *Dev Comp Immunol*. 7: 66-672, 1983.
- Jourdane J, Cheng TC. The two-phase recognition process of allografts in Brazilian strain of *Biomphalaria glabrata*. *J. Invertebr. Parasitol*. 49: 145-158, 1987.
- Kim DE, Dolle MET, Vermeij WP, Gyenis A, Vogel K, Hoeijmakers JHJ. Deficiency in the DNA repair protein ERCC1 triggers a link between senescence and apoptosis in human fibroblasts and mouse skin. *Aging Cell*. e13072, 2019.
- Lie KJ, Heyneman D. Studies on resistance in snails. 3. Tissue reactions to *Echinostoma lindoense* sporocysts in sensitized and resensitized *Biomphalaria glabrata*. *J Parasitol*. 62: 51-58, 1976.
- Lin G, Huang Y-C, Shindel AW, Banie L, Wang G, Lue TF *et al*. Labeling and tracking of mesenchymal stem cells with EdU. *Cytotherapy*. 11: 864-873, 2009.
- Lin H, Stewart CC. Colony formation by mouse peritoneal exudate cells in vitro. *Nat New Biol*. 243: 176-177, 1973.
- Luo Y, Alvarez M, Xia L, Casadevall A. The outcome of phagocytic cell division with infectious cargo depends on single phagosome formation. *PlosOne*. 3: e3219, 2008.
- Mlcochova P, Sutherland KA, Watters SA, Bertoli C, Bruin R, Rehwinkel J *et al*. A G1 like state allows HIV-1 to bypass SAMHD1 restriction in macrophages. *EMBO Journ*. 36: 604-616, 2017.
- Monteil JF, Matricon-Gondran M. Hemocyte production in trematode-infected *Lymnaea truncatula*. *Parasitol Res*. 77: 491-497, 1991.
- Ottaviani E, Franchini A. Ultrastructural study of haemocytes of the freshwater snail *Planorbarius corneus* (Gastropoda, Pulmonata). *Acta Zool*. 69: 157-162, 1988.
- Pan CT. The general histology and topographic microanatomy of *Australorbis glabratus*. *Bull. Mus. Comp. Zool*. 119: 237-299, 1958.
- Pila EA, Sullivan JT, Wu XZ, Fang J, Rudko SP, Gordy MA *et al*. Haematopoiesis in molluscs: A review of haemocyte development and function in gastropods, cephalopods and bivalves. *Dev Comp Immunol*. 58: 119-128, 2016.
- Portet A, Pinaud S, Chaparro C, Galinier R, Dheilly NM, Portela J *et al*. Sympatric versus allopatric evolutionary contexts shape differential immune response in *Biomphalaria/Schistosoma* interaction. *PLoS Pathog*. 15: e1007647, 2019.
- Prokhorova EE, Zhemchuzhnikova EA, Ataev GL. Analysis of ITS1 and ITS2 of ribosomal DNA in populations of *Planorbarius corneus* snails (Gastropoda) from the Leningrad and Kaliningrad oblasts of Russia. *Contemp. Probl. Ecol*. 8: 729-734, 2015.
- Prokhorova EE, Serebryakova MK, Tokmakova AS, Kudryavtsev IV, Usmanova RR, Ataev GL. The analysis of cell composition in the hemolymph of three Planorbidae species (Gastropoda: Pulmonata). *Invert Zool*. 15: 103-113, 2018a.
- Prokhorova EE, Serebryakova MK, Tokmakova AS, Ataev GL. Hemocytes of mollusc *Biomphalaria glabrata* (Gastropoda, Pulmonata). *ISJ*. 15: 346-351, 2018b.
- Prokhorova EE, Usmanova RR, Ataev GL. An analysis of morphological and molecular genetic characters for species identification of amber snails *Succinea putris* (Succineidae). *Invertebrate Zoology*. 17: 1-17, 2020.
- Rodriguez C, Prieto GI, Vega IA, Castro-Vazquez A. Assessment of the kidney and lung as immune barriers and hematopoietic sites in the invasive apple snail *Pomacea canaliculata*. *PeerJ*. 6: e5789, 2018.
- Robbins CS, Hilgendorf I, Weber GF, Theurl I, Iwamoto Y, Figueiredo JL *et al*. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med*. 19: 1166-1172, 2013.
- Salic A, Mitchison TJ. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci. USA*. 105: 2415-2420, 2008.
- Sminia T. Structure and function of blood and connective tissue cells of the fresh-water pulmonate *Lymnaea stagnalis* studied by electron microscopy and enzyme histochemistry. *Z Zellforsch Mikrosk Anat*. 130: 497-526, 1972.
- Sminia T, Baredsen L. A comparative morphological and enzymes histochemical study on blood

- cells of the freshwater snails *Lymnaea stagnalis*, *Biomphalaria glabrata*, and *Bulinus truncates*. *Journal of Morphology*. 165: 31-39, 1980.
- Sminia T, Borghart–Reinders E, Van de Linde AW. Encapsulation of foreign material experimentally introduced into the fresh water snail *Lymnaea stagnalis*: an electron microscopic and autoradiographic study. *Cell Tissue Res*. 153: 307-326, 1974.
- Sminia T, Van der Knaap WPW, Van Asselt LA. Blood cell types and blood cell formation in gastropod molluscs. *Dev Comp Immunol*. 7: 665-668, 1983.
- Souza SS, Andrade ZA. On the origin of the *Biomphalaria glabrata* hemocytes. *Mem Inst Oswaldo Cruz*. 101: 213-218, 2006.
- Sullivan JT. Hematopoiesis in three species of Gastropods following infection with *Echinostoma paraensei* (Trematoda: Echinostomatidae). *Trans. Amer. Micros. Soc*. 107: 335-361, 1988.
- Terry NH, White RA. Cell cycle kinetics estimated by analysis of bromodeoxyuridine incorporation. *Methods Cell Biol*. 63: 355-374, 2001.
- Van der Knaap WP, Loker ES. Immune mechanisms in trematode-snail interactions. *Parasitol Today*. 6: 176-182, 1990.
- Wiley CD, Brumwell AN, Davis SS, Jackson JR, Valdovinos A, Calhoun C *et al*. Secretion of leukotrienes by senescent lung fibroblasts promotes pulmonary fibrosis. *JCI Insight*. pii: 130056, 2019.
- Zeng C, Pan F, Jones LA, Lim MM, Griffin EA, Sheline YI *et al*. Evaluation of 5-ethynyl-2'-deoxyuridine staining as a sensitive and reliable method for studying cell proliferation in the adult nervous system. *Brain Res*. 1319: 21-32, 2010.
- Zink F, Stacey SN, Norddahl GL, Frigge ML, Magnusson OT, Jonsdottir I, *et al*. Clonal hematopoiesis, with and without candidate driver mutations, is common in the elderly. *Blood*. 130: 742-752, 2017.