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RESEARCH REPORT

The influence of trematode infection on the hemocyte composition in *Planorbarius corneus* (Gastropoda, Pulmonata)

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Abstract

Hemocytes are the main effector elements of gastropod anti-trematode defence reactions. Elucidation of morphological and functional characteristics of hemocytes allows for better understanding of gastropod resistance mechanisms. Hemocyte composition of *Planorbarius corneus* revealed types of cells: granulocytes and hyalinocytes, which differ in granularity, nucleus-to-cytoplasm ratio and spreading compatibility. Flow-cytometric analysis suggested the presence of these cell types in the hemolymph of *P. corneus* and showed the differences in granulocyte/hyalinocytes ratio in non-infected snails and snails infected with different trematodes - *Cotylurus sp*., *Notocotylus sp.*, *Plagiorchis sp*. and *Echinostoma sp*. It was also shown that in snails with large shell diameter (34 - 37 mm), the ratio of cell types in the hemolymph is clearly biased in favour of granulocytes.

Key Words: snails, trematodes, granulocytes, hyalinocytes, defense reactions, phagocytosis

Introduction

Hemocytes are the principal effector elements of defence reactions in gastropods. They are involved in all stages of the defence reaction - identification, isolation and elimination of foreign bodies - and also in restoring the mollusc's internal environment after defence reaction (Adema *et al.*, 2000; Connors, 2003). Information on hemocyte morphological types and their functional activity remains fragmentary. Inconsistencies in the current system of hemocyte classification make comparison difficult. In addition, most immunological researches in gastropods have been done only on few species of mollusc, mainly on genus *Biomphalaria*. There is a need to expand the set of molluscs used in such work. *Planorbarius corneus* is a widespread species and an intermediate host for many trematodes (Faltynkova *et al.*, 2004; Brown *et al.*, 2011). It attracts the attention as natural model object for studying hemolymph/ immunological assays (Ottaviani and Cossarizza, 1990; Ottaviani *et al.*, 1993), molecular genotyping (Prokhorova *et al.*, 2015), physiological and ecological research (Otludil *et al.*, 2004; Stepan *et al.*, 2012; Zbikowska *et al.*, 2013). However, the analysis of trematode infection on the hemocyte composition in *P. corneus* has not been done before.

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Materials and Methods

Snails

Planorbarius corneus (n = 275) molluscs were collected from pure water springs in Leningradskaya Oblast (Russia). Molluscs species were identified based on morphological criteria (Gloer, 2002; Alexeev and Tsalolyhin, 2016). The molluscs were kept in 10 - 20 L plastic aquariums filled with a mix of tap water and filtered pond water (1:1) in climatic chamber at 21 ± 1 °C under 12-h light: 12 h dark photocycle. The water was aerated continuously and changed every 3 days. Chalk was used for Ca^{2+} source and pH stabilized at about 7.0 with sodium bicarbonate. All snails were fed on lettuce leaves. Some of the snails were infected with *Cotylurus brevis* (Strigeidae) (n = 10), *Notocotylus ephemera* (Notocotylidae) (n = 12), *Plagiorchis multiglandularis* (Plagiorchiidae) (n = 14) and *Echinostoma spiniferum* (Echinostomatidae) (n = 10). The definitive hosts of all these digenea species are wild ducks. The trematode infection of molluscs was defined by cercarial shedding. Mollusks were put to individually plastic cuvets in the early morning and at the end of afternoon for detection of cercarial

shedding every other day during 4 weeks following collection. The extent of infection was defined by dissection of molluscs after hemolymph collection. For the experiment, molluscs with shell diameter of 22 - 37 mm were selected (most measured 26 - 29 mm).

Hemolymph collection, incubation and sample preparation

For morphological typing of hemocytes of *P. corneus* (n = 107), haemolymph was collected using glass pipettes from the pericardial region of the snail (Sminia and Baredsen, 1980). From 3 to 6 hemolymph samples were done for every mollusc. The number of hemocytes in 1 µl of hemolymph was counted in cell-counting chamber (Cell-Line Associates).

A part of hemolymph specimens were used to prepare fixed smears. Haemolymph was applied on polylysine-coated glasses and cells were allowed to settle in a humid chamber for 30 - 40 min. Then smears were fixed with 4 % paraformaldehyde solution prepared on PBS, rinsed twice by PBS, and stained with Erlich hematoxylin-eosin. Observations of hemolymph smears were used in addition to live hemocyte analysis for hemocytes morphological typing.

To study the hemocytes *in vitro*, hemolymph from individual snails was placed on plastic Petri dishes and incubated in a humid chamber for 4 - 8 h at 22 - 24 °C. During the incubation the specimens were intermittently observed (every 10 - 20 min) using a phase-contrast microscope (Leica DM 5000). For the majority of snails it the total number of hemocytes/µl were counted. Cell counts and size measurements were preformed using ImageScope software (CMA, Russia). Every cell was measured twice - in minimum and maximum diameters, cell areas were calculated.

Flow-cytometric assay

Hemolymph for analysis in the flow cytometer (Coulter Epics Altra, Beckman Coulter) was collected in plastic Eppendorf tubes with 20 mM EDTA. Sample analysis was done immediately with the following cytometric parameters. Forward light-scatter (FS) and side-scatter (PMT1) signals were collected for 30,000 cells from each sample and stored as list mode data files. FS gives a relative indication of cell size, while PMT1 is an indication of complexity, texture or granularity of cells. Gating of the cells was performed to exclude dead cells and debris from subsequent analyses. Ranges for distinguishing the cell groups characterized on different size and complexity have been chosen first for non-infected snails and also were applied for trematode-infected molluscs. In the flow-cytometric analysis of tematode-infection influence on the hemocyte composition number individuals with 26 - 29 mm shell diameter were used including 47 non-infected and 46 trematode-infected molluscs.

Phagocytosis assay

Fluorescein isothiocyanate (FITC)-conjugated *Escherichia coli* and *Staphylococcus aureus* bacteria were used to study phagocytic activity in the hemocytes. The bacteria were stained with FITC (Sigma) according to the method of Coteur *et al.* (2002). Incubation was carried out in the dark at 4 °C for 24 h. Then the material was washed in PBS (1.7mM KH_2PO_4, 5.2mM Na_2HPO_4, 150 mM NaCl, pH = 7.4) and physiological saline solution (0.9 % NaCl). Bacterial cell concentration was 10^7 cells/ml. The bacterial suspension (80 - 100 µl) was injected into the foot of the mollusc (n = 33). A control group of molluscs was injected with buffered saline solution (n = 20). Hemolymph was analysed using a fluorescent microscope (Leica DM 5000) and flow cytometer at three, six and 12 h post injection. For flow-cytometric analysis haemolymph was quenched with 0.5 % trypan blue in PBS (Serva). While FS and PMT1 characteristics were acquired in linear mode, fluorescence intensity at wavelength of 530 nm (PMT2, FITC) was acquired at log scale. This channel was used for the analysis of green fluorescence positive cells. The resulting files were analyzed using Expo32 (Coulter, Hialeah, FL) software. To study phagocytic activity in the haemocytes *in vitro*, hemolymph from snails (n = 22) was incubated with a suspension of the FITC-labeled *St. aureus* bacteria in a humid chamber at 22 - 24 °C. The final bacterial concentration was adjusted to 10^7 cells/ml. Samples were analysed every 10 min during 6 h.

Statistical analyses

Data were analyzed using Microsoft Excel software (Microsoft). Differences between data groups were tested by Student's t-test for independent and dependent samples. Differences were considered as significant at $p < 0.05$. Results are shown as mean percentage and standard deviation. In order to test correlation between distinct criterions Pearson's correlation coefficient (r) was used. The significance was computed using t - test in PAST software (http://folk.uio.no/ohammer/past).

Results

Morphological types of hemocytes

Based on cell morphology, two distinct types of *P. corneus* (n = 107) hemocytes were observed. Majority of cells (70.5 ± 3.1 %) were granulocytes (Figs 1a - d), the spreading cells with dimensions of 9.5 ± 5.9 x 12.95 ± 7.9 µm (cell areas of 174.26 ± 34.18 μm^2) and oval nuclei (diameter 4.1 ± 2.8 x 5.3 ± 2.2 µm). Their average nucleus-to-cytoplasm ratio (N/C) was about 0.12. The internal part of the cell's cytoplasm contained numerous granules and vesicles. Granulocytes form numerous filopodiae and seldom lobopodiae.

The less numerous subpopulation was represented by (24.7 ± 2.3 %) hyalinocytes (Fig. 1d), rounded cells with dimensions 6.1 ± 1.2 x 8.1 ± 1.5 µm (cell areas of 41,79 ± 6.54 μm^2), containing spherical or oval nuclei (diameter 2.6 ± 1 x 3.3 ± 1.3 µm). The average nucleus-to-cytoplasm ratio of these cells was approximately 0.25, and they were capable of forming lobopodiae.

The morphology of some cells (about 3 %) was similar to hyalinocytes (Fig. 1f), but they were smaller (4.5 ± 0.4 µm diameter with average N/C of 0.48.

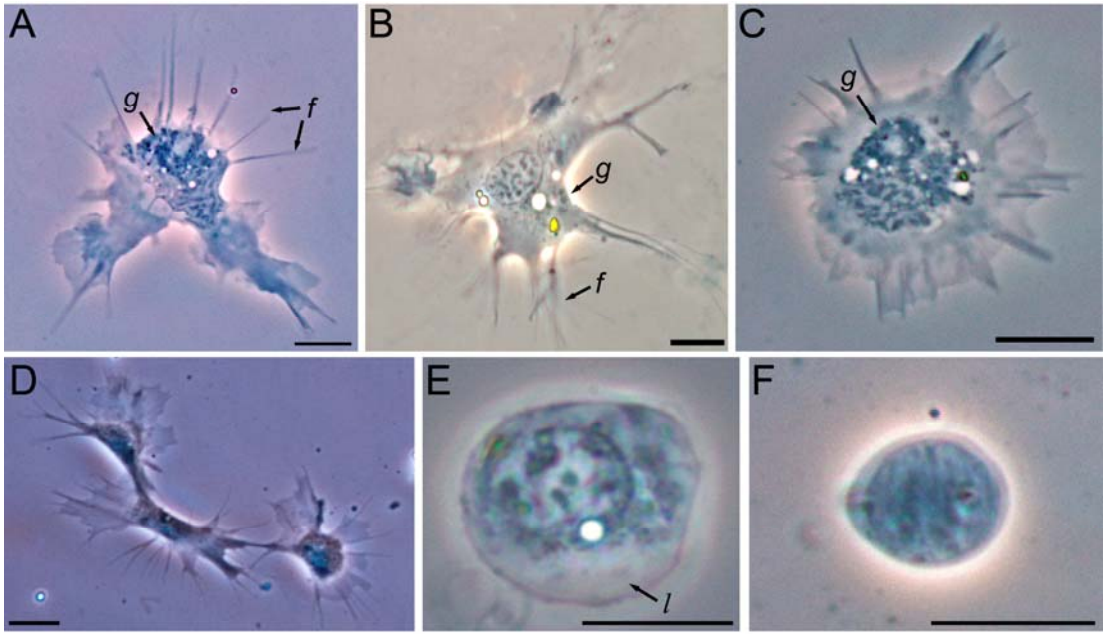

Fig. 1 Hemocytes from *P. corneus.* Two main types of cells with different spreading compatibility and granularity were obtained. Granulocytes (A - D) contains many granules (g) and vesicles in abundant cytoplasm and form numerous filopodiae (f), rapidly spreading across the substrate. After long-term incubation in a humid chamber the majority of the granulocytes change to large cells with nuclei containing large amounts of scattered heterochromatin clumps (B). Hyalinocytes (E, F) have thin cytoplasm and mainly oval or round shape and sometimes form one lobopodia (l). These cells slowly spread on the substrate. Phase-contrast microscopy. Bar = 5 μm.

Pools of hemocytes, detected by flow cytometry

Flow cytometry of the hemolymph of *P. corneus* molluscs detected two populations of hemocytes: small cells with a low number of granules, and larger, more granular cells. In terms of relative dimensions and granularity, these haemocyte populations correspond to the above described granulocytes and hyalinocytes, respectively (Fig. 3).

In non-infected molluscs (n = 47), the hyalinocytes account for 58.5 ± 6.5 % of all hemocytes, and the granulocytes 37.1 ± 7.3 %. However, the cell populations are not segregated evidently (Figs 3e, f).

The correlation between number of circulating hemocytes and snail size

One μl of hemolymph from a non-infected *P. corneus* mollusc contains 439 ± 176 cells (from 215 to 1,089) (Table 1). No significant difference in number of hemocytes in molluscs with different shell diameter was observed. However, correlation analysis of the hyalinocytes/granulocytes ratio and mollusc shell diameter established a reliable inverse correlation ($r = -0.93$, $n = 31$, $p < 0.001$). Nevertheless, hyalinocytes predominate in all groups (Table 1).

The activity of hemocytes

P. corneus hemocytes retain their ability to survive in the humid chamber up to 8 h. Hemocytes from snails which had previously been injected with *E. coli* and *S. aureus* remained viable for 4 - 6 h. During the observation period granulocytes changed their shape. They gradually spread across the substrate, simultaneously moving through it. The hyalinocytes gradually became fixed on the substrate, while their shape remained almost unchanged (Figs 2a, b).Often, granulocytes formed aggregates containing up to 10 cells (Fig. 1c).

When kept in a humid chamber for more than 5 h, large cells with nuclei containing large amounts of scattered heterochromatin lumps formed the majority of the granulocytes (Fig. 1b).

Flow cytometry showed high intensity fluorescence of *P. corneus* hemocytes 3 h post injection with FITC-labelled bacteria.

S. aureus were phagocytosized in 78.5 ± 7.1 % (n = 25) of cases (Figs 4a, b), *E. coli* in 49.3 ± 12.4 % (n = 8) of cases (Fig. 4c). Moreover, *E. coli* were preferably absorbed by hyalinocytes, whereas granulocytes exhibited preference for *S. aureus*. The fluorescent intensity of hemocytes in molluscs of the control group (n = 20) remained at the same level as the intact individuals.

Three h after FITC-labelled bacteria injection fluorescing structures with dimensions (2 - 4 um) significantly greater than the dimensions of the bacterial cells were observed in cytoplasm of the granulocytes (Fig. 2c). Fluorescent structures were found only in small proportion of granulocytes 4 - 6 h post injection. No such structures were observed in the hyalinocytes.

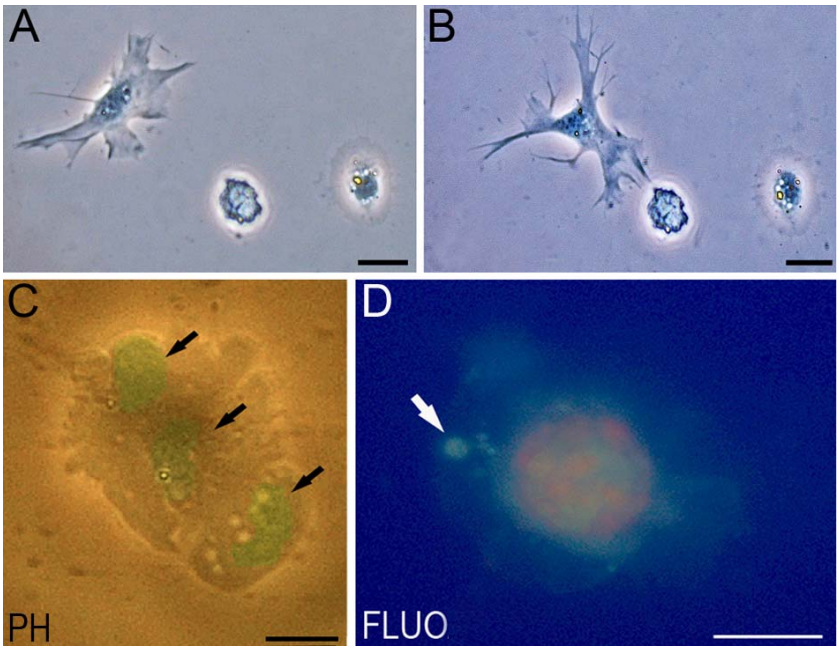

Fig. 2 The functional activity of *P. corneus* granulocytes. A, B) Demonstration of granulocytes motility. Granulocyte (left) changes the shape and moves across the substrate. Hyalinocyte (right) doesn't move and remain almost unchanged. Phase-contrast microscopy. Bar =1 0 µm. C, D) Phagocytosis of *S. aureus* by granulocytes. C) hemocyte of mollusc injected with FITC-marked *S. aureus* suspension 3 h post injection. Fluorescing structures in cells (are indicated by arrows) are phagocytic vacuoles containing partially digested bacteria. D) hemocytes obtained by incubation in vitro with *S. aureus* during 30 min. Phagocytic vacuoles containing bacteria appear in the hemocytes' cytoplasm (are indicated by arrow). PH = phase-contrast microphotography, FLUO = fluorescence photomicrographs. Bar = 10 µm.

In the cytoplasm of hemocytes incubated with bacteria *in vitro* for 15 - 30 min, fluorescent structures with dimensions equivalent to the bacterial cells were observed (Fig. 2d). Following longer incubation periods, large fluorescent granules were observed in hemocytes similar to those described above for hemocytes in molluscs injected with bacteria.

Influence of trematode infection on the ratio of circulating hemocytes types

The ratios of granulocytes to hyalynocytes in trematode-infected molluscs, and non-infected snails were clearly different (Figs 3a - d, represent the individual flow-cytometric profiles). In molluscs infected by *C. brevis* (n = 10), two distinct populations of cells were observed. Hyalinocytes made up 32.9 ± 1.7 % and granulocytes 57.4 ± 8.4 % of all hemocytes (Figs 3a, f). In molluscs infected by *N. ephemera* (n = 12), hyalinocytes and granulocytes comprise 34.1 ± 9.1 % and 56.1 ± 10.6 %, respectively, of all hemocytes (Figs 3b, f). In molluscs infected by *P. multiglandularis* (n = 14), the respective populations of cells were similar to non-infected individuals: granulocytes 31.7 ± 5.1 % and hyalinocytes 56.3 ± 4.9 %. In this case, however, a boundary could be clearly discerned between the granulocytes and the hyalinocytes (Figs 3c, f). In snails infected with *E. spiniferum* (n = 10), hyalinocytes comprised an average of 41.3 ± 8.1 % of hemocytes, and 38.1 ±11.5 % of granulocytes (Figs 3d, f).

Table 1 Number hemocytes and percentage of granulocytes and hyalinocytes in the hemolymph of *P. corneus* snails with different shell diameters according to flow-cytometric analysis.

Shell diameter	22-25 (n=10)	26-29 (n=18)	30-33 (n=10)	34-37 (n=9)
Number of hemocytes in 1 µl of hemolymph	356±212	459±195	462±154	480±172
Percent of hyalinocytes	66.4±5.09	61.74±5.88	54.43±4.44	55.44±1.37
Percent of granulocytes	29.56±4.23	33.6±5.57	40.28±4.98	40.28±6.13

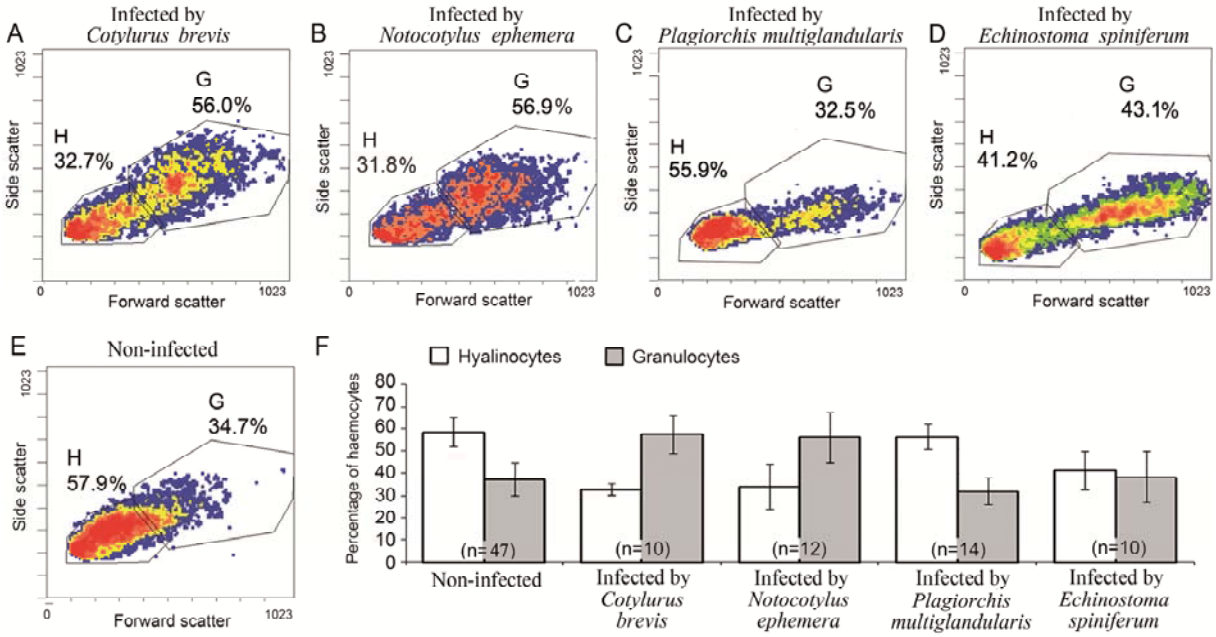

Fig. 3 A - E. Individual flow cytometric profiles of hemolymph from *P. corneus* trematodes infected (A - D) and non-infected (E) snails. Profiles showing a distribution of side scatter (SS, indicates relative granularity) and forward scatter (FS, SS, indicates relative size). 30,000 cells were analyzed from each sample. Two distinct types of hemocytes were detected: hyalinocytes (region H) small cells with a small number of granules, and granulocytes (region G) larger, more granular cells. F. Percentage of granulocytes and hyalinocytes in *P. corneus* none-infected and trematode-infected snails. The hyalinocytes/granulocytes ratio is different in non-infected snails and snails infected by distinct trematodes.

Discussion

The snail-trematode host-parasite system is a commonly used model to study defence reactions of molluscs. Cell reactions such as encapsulation, formation of a paleot around the parasite, and changes in the number of circulating hemocytes were firstly described for molluscs infected by trematodes (Lie and Heyneman, 1975; Galaktionov and Dobrovolsky, 2003). Cell reactions to trematode infection are currently considered as a resistance mechanism in the host-parasite system (Loker, 2010). As such, hemocyte composition and peculiarities of hemocyte activity are useful tools to characterize the immune system of snails and define their resistance mechanisms to the parasite.

Our results suggest the presence of two main cell types in *P. corneus* hemolymph: granulocytes and hyalinocytes. Hyalinocyte and granulocyte populations exhibited distinct morphology, granularity rate, motility, and phagocytosis activity. Flow cytometry analysis of the haemolymph confirmed the data obtained by microscopy. The populations of small, low granular hyalinocytes and large, more granular granulocytes were revealed. Similar subpopulations of *P. corneus* hemocytes have been earlier described by Ottaviani (1983). The above-referenced study described "round cells" and "spreading cells", were labelled by different groups of bioactive polypeptides, and exhibited differences in their organelle composition (Ottaviani *et al.*, 1991; Ottaviani and Franchini, 1988).

Large cells which remained viable for longer period of time than other hemocytes. Morphologically similar cells have been described in the composition of the cellular capsules that form around degenerating sporocysts (Cheng and Galloway, 1970), allografts and xenografts of different snail tissues (Cheng and 1984; Sullivan *et. al*., 1993; Orta and Sullivan, 2000). It has been suggested that some granulocytes become hypertrophied cells in response to pathological changes in the recipient-mollusc's organism, while the rest merge to form megacytes (Jourdane and Cheng, 1987). It has also been suggested that granulocytes have greater resistance to disturbances of homeostasis (Hahn *et al.*, 2001). Metabolites facilitating cell destruction accumulate during long-term incubation of mollusc hemocytes. Large granulocytes (Fig. 1b) appear to be more resistant to the toxic effects of the metabolites than other cell types. It is conceivable that the large granulocytes represent a specialised group of cells involved in encapsulation processes.

The ability of granulocytes to spread across the substrate and to adhere to other cells confirms their principal roles in the process of encapsulating alien bodies (Van der Knaap and Loker, 1990; Loker, 2010). We observed significant variability not only in size, but also in morphology of granulocytes. After

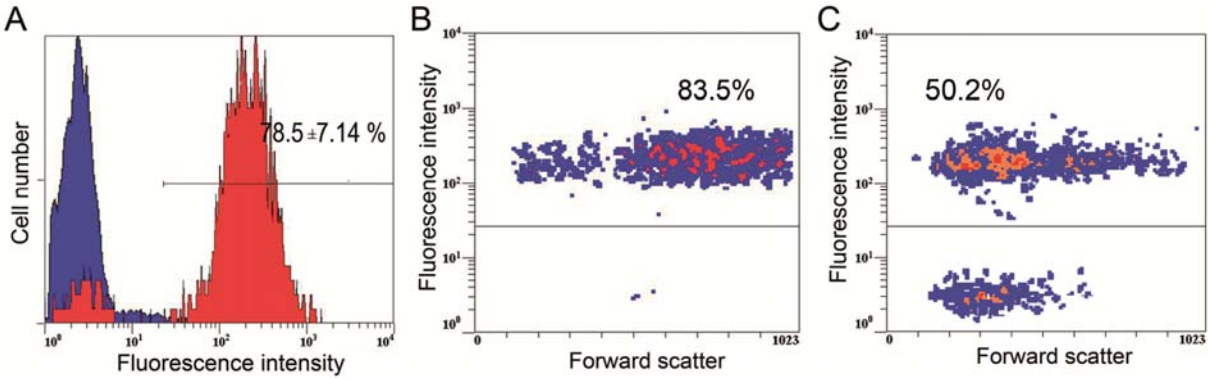

Fig. 4 Phagocytosis of bacteria by hemocytes of *P. corneus* snails injected by bacteria suspension. Flow-cytometric analysis showed high intensity fluorescence of *P. corneus* hemocytes three hr post injection with FITC-labelled bacteria. Fluorescent positive (phagocyted) cells were detected at wavelength of 530 nm (PMT2, FITC). *St. aureus* were phagocytized in 78.5 ± 7.1 % (n = 25) of cases, primarily by granulocytes (B) and *E. coli* in 49.3 ± 12.4 % (n = 9) of cases, primarily by hyalinocytes (C). A) histogram of fluorescent intensity of hemocytes from snails injected with *S. aureus*. B) plots of phagocytosis of *S. aureus*, C) plots of phagocytosis of *E. coli*. Percent of hemocytes which phagocytized bacteria is shown.

several h of incubation, these cells can change their shape, dimensions, and number of pseudopodia. Granulocytes of various forms (flattened and polygonal) have been described in the composition of cellular capsules around transplants and parasites (Byrd and Maples, 1969; Lie and Heyneman, 1976; Loker *et al.*, 1986; Ataev and Coustau, 1999). Morphological changes in granulocytes during incubation graphically illustrate their polymorphic nature. Previously described morphotypes of pulmonate granulocytes probably represented various phases in granulocyte differentiation.

Analysis of phagocytic activity of hemocytes also confirmed the existence of different functional hemocyte populations. Flow-cytometric analysis of the uptake of FITC-conjugated bacteria by hemocytes in *P. corneus* demonstrated a reduction in fluorescence in hemocytes of phagocytised bacteria at 6 - 12 h post injection. Haemocytes rapidly defended the mollusc's internal environment from pathogens. In the cytoplasm of granulocytes in molluscs injected with FITC-labelled *St. aureus*, large fluorescent granules were visualised after 1 - 3 h. These granules are likely to represent phagocytic vacuoles (phagolysosomes) containing partially digested bacteria.

Flow-cytometry analysis and microscopical analysis provided different granulocyte/hyalinocyte ratios. Obviously such different results were due to the low ability of hyalinocytes to spread and adhere to substrates. Also, some of the cells were lost during preparation of monolayers and were not counted.

Bacterial injection leads to a 12 - 19 % increase in the number of *P. corneus* circulating hemocytes. Similar data have previously been obtained for bivalves. Injections of bacteria into mussels and oysters also lead to an increase in the number of circulating hemocytes (Hernroth, 2003; Terahara *et al*., 2006).

Similar data have been obtained for Bivalvia. Introduction of Gram-negative *Vibrio* bacteria into mussels leads to a sharp reduction in the number of hemocytes. However, the relative number of hyalinocytes increases, which may prove that these cells are involved in antimicrobial response (Allam *et al.*, 2001). Gram-positive *S. aureus and* Gram-negative *E. coli* were phagocytised primarily by granulocytes and hyalinocytes respectively, there is functional differentiation between different types of hemocytes depending on type of antigen (Parisi *et al*., 2008). The microbicidal activity of granulocytes has been confirmed by the antimicrobial peptides expression. Defensins and myticins are accumulated in the granulocytes cytoplasm in bacteria-immunised bivalves and ensure the elimination of Gram-positive bacteria and funguses (Mitta *et al.*, 2000a, b).

Hemocytes are involved in all steps of the snail anti-parasite response (Loker, 2010). As such, changes in the concentration of circulating cells can be seen as one of important criterions in defense reactions (Ataev and Polevshchikov, 2004). Immunization of snails by different antigens can induce the activation of hematopoiesis (Ataev *et al.*, 2000; Azevedo *et al*., 2006; Ataev and Prokhorova, 2013; Sullivan and Belloir, 2014). Experiments show that the local cell reaction results the immobilization of large number of cells in the tissue (Avesedo *et al.*, 2006; Prokhorova *et al.*, 2015). Such processes are able to cause the changes in the amount and composition of circulating hemocytes.

Flow-cytometric analysis showed the predominance of hyalinocytes in non-infected snails. However, in molluscs with large shell diameter, the ratio of hemocyte types is clearly biased in favour of granulocytes (Fig. 4). Increases in hemocyte numbers and the predominance of granulocytes in molluscs with large shell diameter indicate that the mollusc's defence systems "acquire" greater resistance to the influence of various antigens during the individual's lifetime.

We have not observed significant differences in the number of hemocytes between non-infected and infected *P. corneus* molluscs. This might be due to the fact that we studied only naturally infected molluscs, for which the time of infection was unknown. Therefore, we were unable to gather data as to the dynamics of the number of hemocytes during the course of trematode infection. Such data, however, have been reported for *Biomphalaria glabrata* (Ataev and Coustau, 1999). During infection of snails by *Echinostoma caproni*, sharp changes in the number of circulating hemocytes were observed only during the first week post infection. Subsequently, their levels declined to just above the number of hemocytes in non-infected molluscs.

Hemocyte populations were more clearly distinguishable in trematode infected *P. corneus* molluscs, suggesting that infection leads to differentiation of the hemocytes involved in cell response. Moreover, the granulocyte/hyalynocyte ratios were different in molluscs infected by trematodes of different types (Fig. 3f). Apparently, differentiation of cell response in pulmonates depends on the species of parasite.

Similar results were described for *Biomphalaria tenagophila* and *B. glabrata* snails infected by *Schistosoma mansoni* (Martins-Souza, 2009). Experimental infection resulted in early reduction of large and medium circulating hemocytes followed by an increase of small hemocytes. Such a response was particularly intense in the parasite-resistant *B. tenagophila*. The authors assumed that hemocyte response is associated with the cellular response of resistant snails against the parasite.

The peculiarity of cell response depends on the different development of the trematode inside the mollusc (Bayne *et al.*, 2001; Galaktionov and Dobrovolsky, 2003). One factor influencing the ratio of circulating hemocytes is the formation of a “paletot” around the parasite (Galaktionov and Dobrovolsky, 2003). As a result of the mutation of cell reactions and the adhesion of a significant number of hemocytes onto the tegument of the sporocyst, the ratio of circulating cells can also change. It also affects the ratio of circulating hemocytes, and, in particular, formation of multilayered hemocyte capsules around the parasites (Ataev and Coustau, 1999).

This study confirms that trematodes closely interact with the internal environment of the mollusc, influencing the ratio of cell types in hemolymph.

Acknowledgments

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