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Functional characterization of *Littorina littorea* (Gastropoda: Prosobranchia) blood cells

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The main functional characteristics of haemocytes from the common periwinkle *Littorina littorea* (phagocytic ability, acid phosphatase activity, cytotoxic properties and generation of reactive oxygen intermediates) were investigated. The blood cells of *L. littorea* demonstrated phagocytic activity for zymozan particles in both plasma and seawater. However, the level of phagocytosis in plasma was higher than in seawater, suggesting the presence of some soluble factors with opsonizing activity for yeast cell walls in the snail haemolymph. Acid phosphatase was detected in haemocytes following phagocytosis of zymosan. Zymosan particles as well as soluble inducers of respiratory burst (mannan, phorbol-myristate acetate, lipopolysaccharide from *Escherichia coli*) were shown to trigger superoxide anion production in *L. littorea* blood cells as evidenced by nitroblue tetrazolium (NBT) reduction. Haemocytes exposed simultaneously to both inducer and the superoxide scavenger enzyme—superoxide dismutase demonstrated a lower ability to reduce nitrobluetetrazolium. Periwinkle blood cells showed plasma-independent cytotoxic activity for human erythrocytes which may be due to the release of superoxide intermediates into the extracellular environment. These results, together with previously obtained data, suggest that haemocytes are the main effectors in the internal defence system of *L. littorea*, with humoral factors playing an accessory role in recognition and elimination of pathogens.

INTRODUCTION

The seven classes of the phylum Mollusca include an impressive array of specialized forms that have colonized a wide range of marine, fresh water and terrestial habitats. Nevertheless, studies on the internal defence system within the Mollusca have been concentrated in a relatively narrow phylogenetic field. With a few exceptions, interest in immune parameters in this phylum has been confined to commercially and medically important species, almost exclusively the bivalves and pulmonate gastropods. It appears that only a few studies have investigated some elements of internal defence in prosobranch gastropods (Cheng & Sanders, 1962; Pemberton, 1970; Ottaviani, 1989; Hooper et al., 2007). As far as haemocytes of the Prosobranchia are concerned, little is known of basic defence functions of these cells. The deficiency of data on immune function within this important group has led to the introduction of *Littorina littorea*, which is a typical representative of the Prosobranchia, as a new model system for comparative immunology (Gorbushin & Iakovleva, 2006). Knowledge of the immunological parameters of *L. littorea* are of great value for tracing the evolution of the internal defence system in the Mollusca, due to the important phylogenetic position of the Prosobranchia. In addition, this snail possesses a very rich fauna of trematode parasites (Werding, 1969) which may serve as an additional model in studies determining the exact mechanisms of the defence system responsible for protection of an invertebrate host against parasite infection.

On the assumption that blood cells play a dominant role in the internal defence of molluscs, we have recently characterized various haemocyte parameters for *L. littorea* (Gorbushin & Iakovleva, 2006). In the present study, an assessment of the functional (phagocytic, reactive oxygen generating and toxic) properties of circulating haemocytes was addressed.

MATERIALS AND METHODS

Mollusc and haemolymph sampling

Common periwinkles, *Littorina littorea* (6–13 years old) were collected during low tide from Kruglaya Bay of the Chupa inlet, (biological station 'Kartesh', Kandalaksha Bay of the White Sea). The animals were maintained in cages until use and supplied with enough food (fucoid sea grass). Prior to the experiments all the snails were examined for trematode infections—only individuals that did not shed cercaria were chosen for the experiments. After haemolymph sampling each snail was dissected and its soft tissues were carefully examined under a dissection microscope for the presence of trematode parthenitae. Infected individuals were excluded from the analysis. Haemolymph was taken by inserting a 25-gauge needle into the buccal sinus of the relaxed snail. The haemolymph was held on ice for no longer then 10 min until use. Relaxation was attained by placing the snails into a tightly closing flask (20 ml) filled with seawater overnight. Haemolymph samples from individual molluscs were used in all experiments. To monitor possible postoperative mortality 30 bled snails were kept in cages for three months with fucoids replaced weekly.

In vitro *phagocytosis assay*

The haemolymph from each of nine snails was divided into eight aliquots to test two media, autologous plasma and filtered seawater (FSW) of natural salinity (24 ‰, ~800 mOsm), at four time points (10, 20, 30 and 45 min). Haemocyte monolayers were prepared on plastic Petri dishes (4 cm diameter) by placing into the centre of each dish a 60 μl-drop of haemolymph diluted 1:2 with FSW (about 60,000 cells). Diluted haemolymph was used in phagocytosis assays to prevent cell aggregation which made accurate cell counts impossible. The dishes carrying the haemolymph drops were left for about 1 h in a moist chamber at room temperature to allow haemocytes to adhere and spread on the plastic surface. The haemocyte monolayers were then gently rinsed at least three times with FSW to remove residual plasma and 60 μl of one of the test media containing zymosan (3.5×10^7 particles per ml) were applied to the monolayers. Following incubation in the humid chamber at 14°C the haemocytes were rinsed in FSW to remove non-ingested particles, fixed with 4% paraformaldehyde, mounted in 50% glycerol and examined using phase-contrast microscopy. The percentage of haemocytes that phagocytosed at least one particle (phagocytosis index; PI) was determined by counting 400 cells in several fields of view.

In a second experiment, the relationship between PI and the proportion of acid phosphatase (AP) positive cells obtained from 12 individuals were evaluated at four time points (20, 30, 60 and 90 min). Preparation of the haemocytes was as described above for phagocytosis in plasma, however, fixed monolayers were stained using the protocol described by Lojda (1962) for localization of intracellular acid phosphatase activity. Briefly, haemocyte monolayers were fixed with a mixture of equal volumes of 4% paraformaldehyde and 96% ethanol for 10 min and then incubated for two hours at room temperature in a mixture containing the following two components: solution A, 5 mg naphthol-AS-phosphate previously dissolved in 500 μl dimethylformamide mixed with 40 ml of ice-cold acetate buffer pH 5.2 and solution B, 8 drops of 4% $NaNO_2$ mixed with an equal amount of pararosanilin. Enzyme-positive sites stained red. The cell nuclei were counterstained with Mayer's haematoxylin.

Means of the phagocytosis index were compared using the paired two-tailed *T*-test. The test dealt with *arcsin*-transformed individual percentage estimates. On the figures re-transformed means and 95% confidence limits are shown.

Assay for measuring ROS production

To determine whether stimulation of the snail haemocytes resulted in the production of reactive oxygen species (ROS), a nitroblue tetrazolium (NBT) assay was used. All stock solutions were prepared in Tris-buffered saline (TBS): Tris 25 mM; pH 7.5, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 320 mM NaCl. Three aliquots of equal volume (100 μl) from each haemolymph sample (N=16) were placed in wells of a 96-well microtitre plate (strip format): one well was designated as experimental and the two others were for control measurements. After centrifugation (360*g*, 5 min) 20 μl of NBT-stock solution (final concentration 0.2 mg/ml) were added to each well, then the experimental wells received 40 μl of the inducer to be tested. The first control well was assigned for evaluation of the basal ROS production rate and therefore received the same volume of inducer-free TBS. The third blood cells aliquot served as a negative control to estimate NBT reduction rate in the presence of the ROS scavenger enzyme—superoxide dismutase (SOD), each well for this control received 30 μl of SOD stock solution, final enzyme concentration being 2 mg/ml, and was incubated for 30 min prior to adding the NBT and corresponding test inducer. To compensate for the differences in total volumes the same volume of TBS was added to both experimental and the first control wells. Final concentrations of the inducers tested were as follows: bacterial lipopolysacharide from *Escherichia coli* strain B 055 (LPS) 0.25 mg/ml, mannan from *Saccharomyces cerevisiae* (MAN) 10 mg/ml, phorbol myristate acetate (PMA) 5 μg/ml and zymozan A (ZYM) 3.5×10^7 particles per ml. All the inducers tested were purchased from Sigma Chemical Co. (St Louis, USA). After 60 min of incubation with NBT and inducer, the plates were centrifuged as described above, wells were rinsed once with TBS, centrifuged and filled with 30 μl of 96% ethanol. Once the wells were dry, 140 μl of dimethylsulfoxide (DMSO) and 120 μl 2M KOH were added and left for 1 h, to solubilize the reaction product. After centrifugation of the plate, the optical density (OD) of the supernatant (200 μl) was measured on an EL301 strip-reader (Bio-Tek) with a 595 nm filter. Paired two-tail T-tests were used to compare control and experimental mean estimates. The OD-indexes shown in the figures were counted as following: the OD for the experimental well (inducer-stimulated ROS production) was divided by OD of corresponding first control well (basal ROS production). The comparisons between mean OD-indexes obtained for different inducer compounds were performed using two-tail T-test for independent measurements.

Cytotoxic assay

To estimate the cytotoxic capabilities of periwinkle haemocytes, human erythrocytes (blood group AB; donor A.G.) were used as target cells. One millilitre of red blood cell suspension (2 ml of blood in 10 ml of sterile Alsever's solution; stored at 4°C) was transferred to a 15 ml centrifuge tube and the cells washed with PBS (4 centrifuge passages: 1500*g*, 10 min). Then, 40 μl of dense erythrocyte sediment were resuspended in 460 μl of FSW and kept on ice until use, (no longer than 4 h). Haemocyte preparations were made as follows: individual samples (100 μl) of haemolymph from ten snails were added to the wells of a 96-well microtitre plate (strip format), two-fold serially diluted with FSW and centrifuged (360*g*, 5 min). After washing with FSW (4 centrifuge passages: 360*g*, 5 min) 20 μl of stock erythrocyte suspension was added to each well (final total volume 150 μl) and left at 12°C. After 2 h of incubation, the wells were gently pipetted to mix the contents, the plates were centrifuged and the optical density (OD) of 100 μl of the supernatant measured using an EL301 strip-reader (Bio-Tek) with a 490 nm filter and 100 μl of FSW as a blank. The FSW and the corresponding individual plasma (cell-free haemolymph) were used as

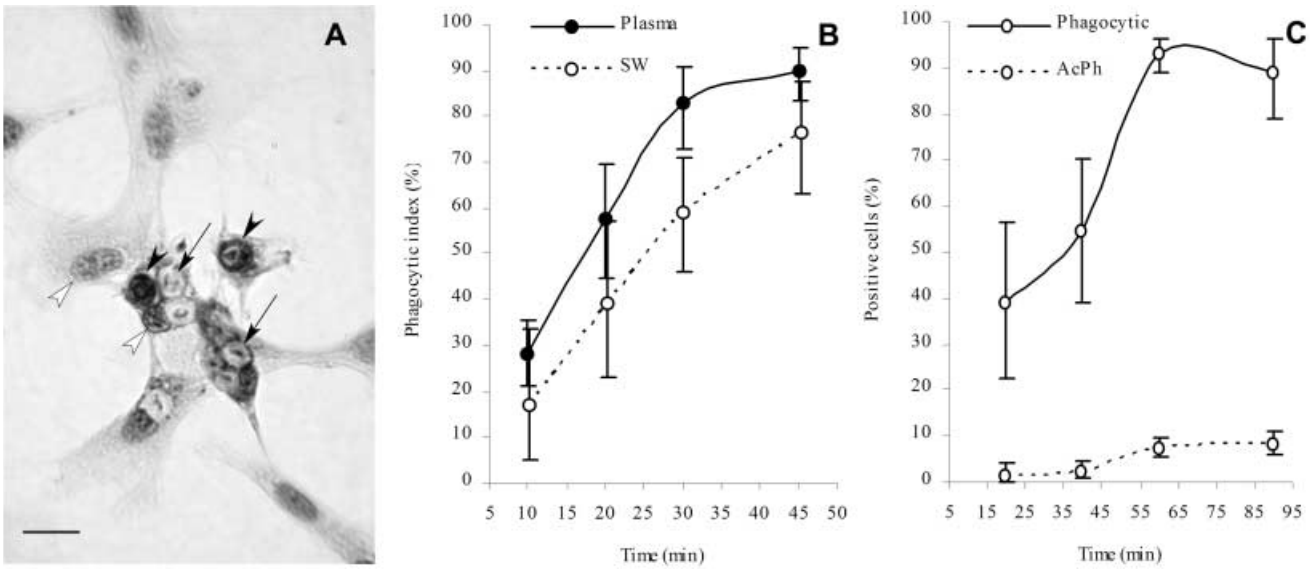

Figure 1. Phagocytosis of zymozan particles by *Littorina littorea* haemocytes; (A) microphotograph of haemocyte monolayer stained with Mayer's haematoxylin, nuclei (white arrowheads), internalized zymosan particles (arrows) and AcPh-positive phagosoms (black arrowheads) are shown; (B) phagocytic index in plasma and seawater as a function of time; (C) proportion of phagocytic and acid phosphatase positive (AcPh) cells as a function of time. Scale bar: A, 10 μm.

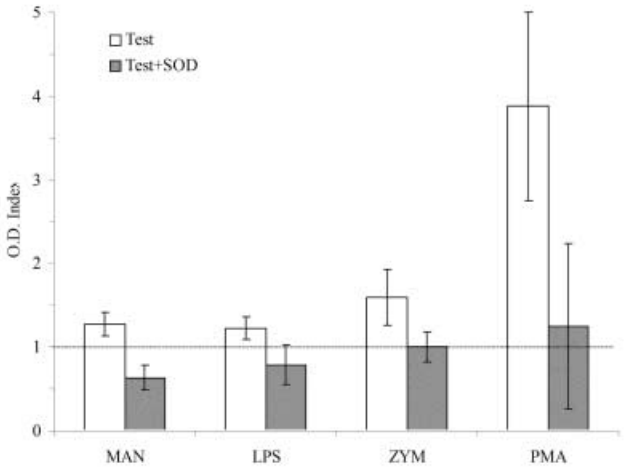

Figure 2. Reactive oxygen species generation by *Littorina littorea* haemocytes exposed to different inductors: (mannan (MAN), lipopolysacharide (LPS), zymozan (ZYM), phorbol myristate acetate (PMA)) in absence (TEST) or in presence of superoxide dismutase (TEST+SOD); dotted line—baseline response of resting cells.

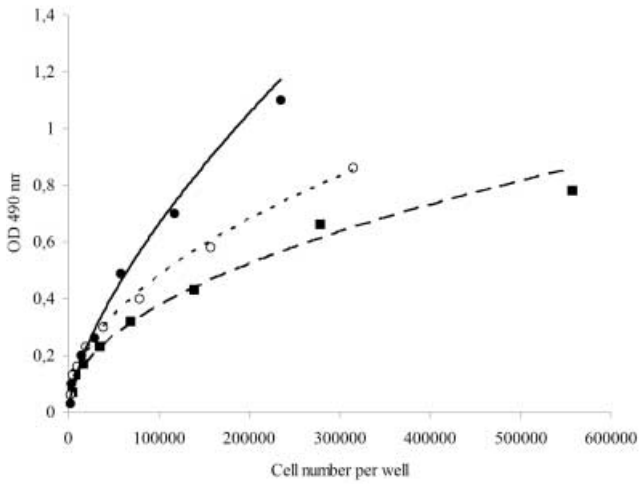

Figure 3. Amount of haemoglobin released from erythrocytes as a function *Littorina littorea* haemocyte number; for simplicity, data recorded from only three individuals are shown.

controls. Additionally, an aliquot of each individual haemolymph sample (20 μl) was fixed with the same volume of 4% paraformaldehyde and the cell concentration was estimated using a Goryaev's haemocytometer. Thus, the haemocyte number in the well (n cells) and the corresponding optical absorption (OD) by haemoglobin released from erythrocytes were used in the correlation analysis.

RESULTS

Depending on individual snail size, the method used enabled the collection of about 400–1500 μl of periwinkle haemolymph. As a rule, the samples were sterile and were never contaminated with mucus and (or) extravisceral liquids. After anaesthesia the bled snails quickly recovered, after about 15 min they restored movement activity and in about an hour regained feeding. There was no mortality attributed to the method used for bleeding the snails. Moreover, when observed for several months, no distinct morphological or behavioural postoperative abnormalities were found.

Phagocytic activity in vitro

Plasma of *Littorina littorea* showed a prominent opsonizing effect on phagocytosis of zymozan particles (Figure 1A,B). After 20 min the phagocytosis index in plasma was significantly ($P<0.05$) higher than in FSW (about 55 and 35% respectively). After 30 min the PI measurements in the plasma stabilized and were similar to the 45 min point (about 85%), however, both were significantly ($P<0.05$) higher then in FSW. Acid

phosphatase activity was found in a maximum of 10% (mean 8%) of phagocytic cells (Figure 1C).

Reactive oxygen species production

To determine whether enhancement of ROS production by *L. littorea* haemocytes occurred in response to soluble stimuli—bacterial LPS, mannan and phagocytic stimulation with zymozan particles, the *in vitro* NBT reduction experiment was carried out. In control preparations, when haemocytes were incubated with NBT without any specific stimulation, a faint background staining was observed. However, all tested stimulants elicited significantly ($P<0.05$) higher levels of total NBT reduction (Figure 2). Co-incubation of cells with zymozan particles caused a very clear reaction resulting in obvious dark blue formazan deposits within the phagosomes. A different pattern of formazan deposition was observed after incubation of haemocytes with soluble stimulants: the cells and plastic surface on the perimeter of the cells were covered with a delicate layer of finest blue granules. The greatest reaction was observed after PMA treatment, whereas after incubation of cells with LPS and mannan the reaction was less obvious. Pre-incubation of cells with the specific superoxide scavenger SOD resulted in a significant ($P<0.05$) decrease in response to all tested substances. It is worth noting, the response of haemocytes in the presence of SOD was reduced to below the baseline response recorded from resting cells.

Cytotoxicity of haemocytes

No haemolysis was found in the control haemocyte-free combinations—FSW and plasma. However, in the wells loaded with haemocytes lysis of erythrocytes was observed after a 40 min incubation. Quantitative measurements of the amount of haemoglobin released within 2 h showed a positive and high correlation with the number of haemocytes placed in the wells (r: 0.90–0.98; $P \ll 0.001$). Experimental snails showed significant individual variation of haemolytic activity (Figure 3).

DISCUSSION

To confirm that plasma of *Littorina littorea* possesses opsonizing properties, its influence on haemocyte phagocytosis was examined using an *in vitro* assay. In the presence of plasma, the PI was significantly higher than in FSW at all time points, except the first one. This result implies that phagocytosis by periwinkle haemocytes involves a plasma-dependent mechanism, possibly a lectin-carbohydrate recognition. Both exogenous plant lectins (Schoenberg & Cheng, 1982; Boswell & Bayne, 1985; Mullainadhan & Renwrantz, 1986) and endogenous lectins, purified from haemolymph or other tissues of several molluscs (Renwrantz & Stahmer, 1983; Fryer et al., 1989; Richards & Renwrantz, 1991) have been shown to facilitate the process of phagocytosis in these invertebrates. The possible mechanism of their actions is the linking of carbohydrate determinants on the surfaces of the haemocyte and target cell or the alteration of the effector cell surface properties (Boswell & Bayne, 1986). The zymosan-binding factors affecting phagocytosis of haemocytes in *L. littorea* lack any agglutinating activity (Iakovleva et al., 2001). A similar phenomenon of clearance of human blood group B erythrocytes from circulation of *Helix pomatia* by non-agglutinating factors was described by Renwrantz & Mohr (1978). Nevertheless, phagocytosis of zymosan by *L. littorea* haemocytes did occur even in the absence of plasma factors. This fact suggests the involvement of cell-associated receptors in the process as described for oyster (Vasta et al., 1982) which ensures the efficient elimination of non-self material in *L. littorea*.

It can be concluded from the analysis of phagocytosis kinetics that the stabilization of PI occurs at 45 minutes in both plasma and FSW. The maximum value of PI was about 85% and 70% for plasma and FSW, respectively. This indicates the presence of a non-phagocytic cell subpopulation in haemolymph of *L. littorea* that may correspond to previously described juvenile haemocytes (Gorbushin & Iakovleva, 2006). The percentage of juvenile haemocytes averages 15% and therefore agrees with the proportion of non-phagocytic cells for plasma exposed haemocyte monolayers.

The low percentage of acid phosphatase-positive haemocytes may be due to the relatively large size of phagosomes occupying almost the total volume of the cytoplasm and, more importantly, to the low number of preformed primary lysosomes. The preformed lysosomes are of small size and appear as faint background AP-staining throughout the cytoplasm of unstimulated haemocytes. If this was the case, the AP concentration in each phagolysosome may have been too low to be detected with the technique employed.

The results suggest that some soluble factors and zymozan particles stimulate a SOD-sensitive nitroblue tetrazolium (NBT) reduction in *L. littorea* haemocytes, indicating the generation of superoxide anions. A faint background staining of unstimulated (control) haemocytes was probably due to activation of the cells by experimental manipulation. Similar basal production of ROS without any additional stimulation was described for *Lymnaea stagnalis* (Dikkeboom et al., 1987). Both the intensity of ROS-generation and the way of ROS production (intra- or extracellular) demonstrated by *L. littorea* haemocytes depended on the nature of the stimulus.

Extracellular formazan deposits occurred after treatment with PMA, LPS and mannan and intraphagosome deposits following exposure to zymozan particles. The different form and location of formazan deposits may reflect the different ways of intracellular targeting of activated NADPH-oxidase that is known to be the main enzyme responsible for superoxide anion formation. According to a recent study on mammalian phagocytes (DeLeo & Quinn, 1996) NADPH-oxidase represents a multicomponent enzyme consisting of at least five subunits and several accessory proteins. In resting cells, two of five subunits are located in the membranes of specific vesicles and the other three exist in the cytosol as a complex. Upon phagocytosis, due to particular signal transduction sequel, the cytosolic complex migrates toward the membrane complex to assemble an active oxidase (Babior, 1999). In the case of phagocytosis, oxidase-bearing vesicles are targeted predominantly to the phagosome membrane (DeLeo et al., 1999) but when a cell is exposed to a

soluble stimulus, the vesicles fuse with the plasma membrane resulting in secretion of superoxide into the extracellular environment (Kobayashi et al., 1998).

The most pronounced cell response was detected following treatment of haemocytes with PMA. Phorbol myristate acetate being a synthetic analog of diacylglycerol apparently causes the NADPH-oxidase activation by direct stimulation of protein kinase C (Liu & Heckman, 1998) which is able to phosporylate the proteins from the oxidase cytosolic complex and so lead to its translocation to the membrane (Nauseef et al., 1991; Bey et al., 2004). Zymozan (yeast cell wall particles) which is rich in mannan, is known to elicit superoxide production in blood cells of other gastropod molluscs: *Lymnaea stagnalis*, *Biomphalaria glabrata*, *Helix aspersa* (Dikkeboom et al., 1987, 1988; Hahn et al., 2000). In the present study, formazan deposits were found also on the surface of plasma membrane of the mannan stimulated haemocytes. This finding supports the idea that these cells have receptors for mannose-containing structures. On the other hand, the reaction of haemocytes to the LPS stimulation illustrates a pre-adaptation of the *Littorina littorea* defence system for elimination of bacteria and suggests the presence of LPS-binding receptors on the membrane of the haemocytes.

The haemocyte-mediated haemolytic assay used in this study has been rarely used for investigating mollusc internal defence. The principal limitation of the technique is difference between the osmotic pressure of mammalian blood and the haemolymph of some molluscs. Indeed, in all the freshwater snails studied up to now (*Biomphalaria*, *Bulinus*, *Planorbis*, *Lymnaea*, *Viviparus*) the physiological osmolality of internal fluids varies between 110 and 130 mOsm (Adema et al., 1992) in freshwater bivalves (*Anodonta*, *Unio*)—about 40 mOsm (own unpublished data) and these values are too low to employ non-fixed erythrocytes (physiological range 290–320 mOsm) as target cells. Under these conditions, the red blood cells are lysed due to the hypo-osmotic stress regardless of haemocyte-mediated toxicity. However, erythrocytes are much more resistant to a hyper-osmotic conditions and were successfully employed in the cytotoxic assay using FSW (800 mOsm) for *L. littorea* haemocytes. A similar approach was used to evaluate humoral- and cellular-mediated cytotoxicity of blue mussel haemolymph (Wittke & Renwrantz, 1984; Leippe & Renwrantz, 1988).

A significant correlation was found between the concentration of released haemoglobin and the periwinkle haemocyte number in the test wells. This haemocyte-dependent haemolysis provides strong evidence that *L. littorea* blood cells secrete lytic factor(s), which is (are) toxic for 'non-self' cells. The phenomenon of cytotoxicity has been reported for many mollusc species, but the majority of studies have dealt with bivalves (Yoshino & Tuan, 1985; Chu, 1988). Nevertheless, round 'lymphocyte-like' cells from the haemolymph of the pulmonate snail *Planorbarius corneus* have been shown to trigger apoptosis in K-562 cells (Franceschi et al., 1991; Monti et al., 1992). Cytotoxic peptides with antitumor activity have been isolated from albumen gland of the opistobranch mollusc *Dollabella auricularia* (Iijima et al., 2003). The observed cytotoxicity of *L. littorea* haemocytes appears to represent an inducible secretion of factors capable of destroying the human erythrocyte membrane. These factors are not secreted constitutively because cell-free plasma did not show any haemolytic activity. Two different mechanisms may be presumed to underlie the observed phenomenon: the first is binding of cytotoxic protein to the erythrocyte surface accompanied by transmembrane pore formation (Hubert et al., 1997) and the second is over-oxidation of membrane lipids by reactive oxygen and nitrogen radicals leading to membrane destabilization and destruction (Hahn et al., 2001a,b). In view of the ability of periwinkle haemocytes to produce ROS extracellularly discussed above, the latter option appears probable. The ROS production appeared to be triggered by erythrocyte phagocytosis which occurs during the incubation of haemocytes with red blood cells. Similarly, generation of ROS by haemocytes of *Lymnaea stagnalis* (Adema et al., 1994) and *B. glabrata* (Hahn et al., 2001a) has been shown to play a role in trematode sporocyst killing. The high variability of individual cytotoxicity shown in the present study may be explained by the different physiological state of the snail internal defence system that appears in the level of haemocyte pre-activation and maturity of the haemocyte population. In a recent study (Gorbushin & Iakovleva, 2006) we showed that a significant variability of individual haemogram parameters exists in *Littorina littorea* from the White Sea.

Haemocytes of *L. littorea* lysed human erythrocytes under plasma-free conditions. These results confirm that humoral factors, even though they play a role in the haemocyte phagocytosis, are not required specifically for haemocyte-mediated cytotoxicity. This finding combined with previously published data (Iakovleva et al., 2001) on the absence of haemagglutinating activity in *L. littorea* plasma indicates that humoral factors may not play a primary role in the internal defence of snails. Although they are obviously important participants in self–nonself discrimination and opsonin-dependent phagocytosis, key effectors of *L. littorea* internal defence are haemocytes, possessing well-developed phagocytic reactions, a potent ROS-generating system as well as cytotoxic activity.

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