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Effects of repeated hemolymph withdrawals on the hemocyte populations and hematopoiesis in *Pomacea canaliculata*

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ABSTRACT

Pomacea canaliculata is a freshwater gastropod considered an invasive pest by several European, North American and Asiatic countries. This snail presents a considerable resistance to pollutants and may successfully face stressful events. Thanks to the unusual possibility to perform several hemolymph collections without affecting its survival, *P. canaliculata* is a good model to study the hematopoietic process and the hemocyte turnover in molluscs. Here we have analyzed the effects of repeated hemolymph withdrawals on circulating hemocyte populations and pericardial organs, *i.e.*, the heart, the main vessels entering and leaving the heart and the ampulla, of *P. canaliculata*. Our experiments revealed that the circulating hemocyte populations were maintained constant after 3 collections performed in 48 h. The tissue organization of the heart and the vessels remained unaltered, whereas the ampulla buffered the effects of hemolymph collections acting as hemocyte reservoir, and its original organization was progressively lost by the repeated hemolymph withdrawals. The hematopoietic tissue of *P. canaliculata* was evidenced here for the first time. It is positioned within the pericardial cavity, in correspondence of the principle veins. Mitoses within the hematopoietic tissue were not influenced by hemolymph collections, and circulating hemocytes never presented mitotic activity.

1. Introduction

Similarly to all protostomian and deuterostomian invertebrates, molluscan immunity relies on both cellular and humoral components. Molluscan immune competent cells are frequently retrieved in the hemolymph and are usually referred to as hemocytes. Several morphologies of molluscan hemocytes have been described so far, but numerous discrepancies exist across and within taxa, suggesting a high degree of heterogeneity and species-specificity of the hemocytes among molluscs [1]. The process of hemocyte regeneration, *i.e.*, the hematopoiesis, occurs with different timing and in different sites in invertebrates. For instance *Drosophila melanogaster* present hematopoiesis only during embryonic and larval period, while adult flies can only rely on the hemocytes produced before metamorphosis [2]. In crustaceans, the hematopoietic process occurs continuously also in adults, and hematopoietic districts as well as hematopoietic cytokines have been described [3,4]. As observed in crustaceans, also molluscs present a continuous hematopoiesis [5], but the information concerning hematopoietic sites and the molecules involved is much less circumstantiated than in crustaceans and insects [6].

Hints of hematopoiesis have been retrieved in the white body of cephalopods [7–9] and in bivalves [10,11]. Among gastropods, the presence of an amebocyte producing organ (APO) has been described in the Heterobranchian freshwater gastropods *Biompahalaria glabrata* [14] and *Planorbarius corneus* [12]. Conversely, in the Heterobranchian land slug *Incilaria fruhstorferi*, hematopoiesis has been suggested to occur throughout the connective tissue and the vascular system, in absence of a well-defined hematopoietic organ [13]. Ultrastructural studies on the APO region of *B. glabrata*, have been performed 30 years ago [14] and described small clusters of cells (ameboblasts) resting on the epithelial cells lining the pericardium. In more recent times, *in vitro* experiments have been carried on dissected APO, and described a region corresponding to the anterior wall of the pericardial sac containing irregularly distributed follicles of hematopoietic cells. *B. glabrata* APO describes a sinus between the pericardial and mantle epithelia, and was intimately connected with other components such as heart, mantle and kidney [15].

Pomacea canaliculata (Gastropoda, Caenogastropoda) is a freshwater gastropod receiving considerable attention as a model from different fields of research, *e.g.*, eco-toxicology [16],

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parasitology [17] and comparative neurobiology [18]. We have recently morphologically and functionally described the hemocytes of *P. canaliculata*, and demonstrated that two principal populations are observed within the hemolymph [19]. The two populations that have been described are represented by small (Group I) and large (Group II) hemocytes. Group I hemocytes are the minority of circulating cells and have all the morphological features of prohemocytes, whereas Group II hemocytes may display an agranular or a granular cytoplasm, with agranular cells being the most represented cell morphology. Even though it is widely assumed that pro-hemocytes represent a juvenile form of hemocytes differentiating into both non granular and granular hemocytes [6], definitive data demonstrating this assumption have not been presented, yet. Hemolymph can be collected from *P. canaliculata* snails without killing them [19], and thus *P. canaliculata* is a suitable model for analyzing which are the effects of repeated withdrawals on snail survival rate and hemolymph composition. Also, the identification of hematopoietic regions of *P. canaliculata* would provide significant information about the hematopoiesis in molluscs. This information may prove of relevance for comparative studies that still lack several details about the location and molecular regulation of molluscan hematopoiesis [6] and could reveal fundamental for the development of new hemocyte lineages [15,20] or the discovery on new cytokines [4].

In the present article we analyzed the changes of circulating hemocyte populations after repeated hemolymph collections and we described the distribution of hematopoietic cells in *P. canaliculata*. Special attention was dedicated to the morphological changes intervened after repeated bleedings in circulating hemocytes and within the components hosted into the pericardial cavity: the heart, the anterior and posterior aortas (main vessels leaving the heart), the ctenidial/pulmonary and renal veins (main vessels entering the heart) and the ampulla (a saccular vesicle located along the anterior aorta route, just beside the heart).

2. Materials and methods

2.1. Animals

P. canaliculata specimens were purchased from T.A.F. Trans Aquarium Fish srl (Italy) and maintained in aquaria filled with aerated freshwater at 24 ± 1 °C. Animals were fed *ad libitum* with vegetables and 10–20% of aquarium content was regularly changed. Adult snails bred effectively and were grown in laboratory conditions.

2.2. Hemolymph withdrawal

In order to obtain a clear hemolymph the animals were kept unfed in a separate tank since 24 h before the first hemolymph withdrawal up to the end of the experiment. In repeated withdrawal experiments, the hemolymph was collected up to 4 times from the same snails allowing 24-h intervals, *i.e.*, one hemolymph collection at time 0 and then after 24, 48 and 72 h for a total of 4 collections [21]. The hemolymph was withdrawn by applying a gentle and continuative pressure onto the operculum, dropping 2 ml of hemolymph within an ice-cold tube in order to prevent cell adhesion and clotting [19]. Immediately after the collection, 1 volume of the anti-clotting solution, 1% (W/v) L-Cystein in freshwater snail solution, pH 8 [19], was added to the hemolymph and maintained at room temperature (RT). After each withdrawal the cell density was evaluated with the hemocytometer.

Three independent set of experiments were performed. For each set of experiments, 3 snails were withdrawn 4 times in 72 h. After the end of the collection protocol, the snails were kept in separated tanks and their mortality was checked daily. The protocol resulted unharmful for the snails since their mortality rate was identical to those registered in normal storage tanks. Moreover, experimental snails kept in behaving and breeding normally during and after the experiments.

2.3. Differential count of circulating hemocytes

Immediately after each withdrawal 100 μl of hemolymph diluted with anti-clotting solution were cytocentrifuged at 300 rpm for 2 min ("Cytospin II", Shandon Instrument, UK) onto slides. The cells were fixed and stained with "Diff-Quik®" kit (BioMap snc, Italy) for morphological observations, rinsed with distilled water, mounted in "Eukitt®" mounting medium (Kindler GmBH, Germany) and observed under an "Olympus BH-2" light microscope (Olympus Corporation, Japan) equipped with a "DS-5M-L1 Digital Sight Camera System" (Nikon, Japan). For each sample, the cell morphology was carefully analyzed following the morphological description of Accorsi et al. [19], and the number of hemocytes belonging to Group I or Group II was counted. The percentages of Group I and Group II hemocytes, respectively, were calculated on a basis of 100 cells as a minimum. The value of the nucleus/cytoplasm (N/C) ratio and the cell size, used in distinguishing the Group I and II morphotypes [19], were obtained with "ImageJ 1.32j" (Wayne Rasband, National Institute of Health, USA).

2.4. Morphological analysis of P. canaliculata pericardial cavity organs

Six h after either the second or the fourth hemolymph withdrawal the animals were anesthetized by putting them in an ice-water bath for 10 min and the pericardial cavity components were dissected under a stereomicroscope. The morphological analysis of the heart, the anterior and posterior aortas, the ctenidial/pulmonary and renal veins, and the ampulla, was performed on both control and withdrawn snails. After the dissection, the components were immediately fixed in Bouin's fixative solution for 6 h at RT, dehydrated and included in paraffin-beeswax including medium. The 7-μm thin sections were stained by Azan-Mallory's staining. The Azan-Mallory's trichrome staining combines azocarmine G, orange G and aniline blue allowing the discrimination of the cell nuclei and the extracellular components. This staining may be used during morphological analysis of several tissues because it displays different colors on the basis of the targeted components. The nuclei are evidenced in red to purple color, the cell cytoplasm is pale red, the collagen fibers are colored in deep blue and muscle fibers in red. The observations were performed under an "Eclipse 90i" light microscope (Nikon) equipped for differential interference contrast microscopy and connected to a DS cooled camera head "DS-5Mc". Images were acquired using the "Act-2U" software (Nikon).

2.5. Immunohistochemical staining of the pericardial cavity organs

The sections of the pericardial cavity organs were immunostained following the standard protocol for diaminobenzidine (DAB)-revealing methods [22]. The endogen peroxidases were inactivated by incubating the rehydrated slides in a 0.3% methanol solution in H_2O_2 for 30 min at RT. Non-immune serum was used before the specific primary antibody in order to saturate non-specific binding sites. The incubation with the primary antibody anti-phospho(Ser10)-histon H3 [p(Ser10)-H3] rabbit pAb (Euroclone, Italy) diluted 1:100 in PBS [23] was performed overnight at 4 °C. The anti-p(Ser10)-H3 pAb is a specific marker for mitotic cells [24]. Developed in 1997 for evidencing chromatin modifications in