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Discrimination of haemocytes of *Astacus leptodactylus* (Decapoda: Crustacea) by differential spreading and cytochemical staining

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Abstract

Haemocytes are important components of the immune system of arthropods. A simple method is described to differentiate the three major types of haemocytes of the decapod crustaceans *Astacus leptodactylus*, the hyaline cells, the granular and semi-granular haemocytes. Haemocytes are fixed after attachment to a microscopic slide in glutaraldehyde, rinsed in ethanol and incubated in DOPA or dopamine, the substrate of the enzyme phenoloxidase. Due to their different melanin staining and spreading capabilities the haemocyte types can easily be recognised and counted comparing their bright field and phase contrast microscopic image.

Zusammenfassung

Differenzierung von Haemozyten von *Astacus leptodactylus* (Decapoda: Crustacea) mittels Spreitung und Färbung – Haemozyten sind bedeutende Komponenten des Immunsystems bei Arthropoden. Eine einfache Methode zur Differenzierung der drei Haupttypen der Haemozyten des decapoden Krebses *Astacus leptodactylus*, den Hyalinen Zellen sowie den Granulären und Semi-Granulären Zellen, wird beschrieben. Die an einem Objektträger angehefteten Haemozyten werden in Glutaraldehyd fixiert, mit Ethanol abgespült und in DOPA oder Dopamin inkubiert. Aufgrund der unterschiedlich intensiven Melaninbildung und Spreitung können die Haemozytentypen durch Vergleich in Hellfeld und Phasenkontrast leicht erkannt und gezählt werden.

1. Introduction

The haemocytes of decapod crustaceans comprise three main types: the hyaline, the semi-granular and the granular cells (Bauchau 1981, Hose et al. 1990, Lanz et al. 1993). The differentiation of these haemocyte types has been difficult and some scientists still consider it to be impossible without electron microscopic investigations. Haemocytes are responsible for different basic immune defence processes in crustaceans, e.g. production and storage of antibacterial substances (Smith & Chrisholm 1992, Xylander et al. 1997), activation of melanisation processes (Smith & Söderhäll 1986, Söderhäll & Smith 1986 a, b, Johansson & Söderhäll 1989), haemocytic clot formation, opsonisation of foreign material (Söderhäll

1981, 1982), phagocytosis and multicellular encapsulations (NEVERMANN et al. 1996). As these immune reactions can be assigned to specific haemocyte types, a rapid method for discrimination of haemocytes in vitro would be a valuable prerequisite for further investigations. We therefore worked out a simple method to differentiate crustacean haemocytes in vitro using their different spreading and cytochemical staining capabilities.

2. Materials and methods

Specimens of the Turkish freshwater crayfish *Astacus leptodactylus* were reared in large oxygenated basins at 10 – 13 °C water temperature. All animals used in this study were adult with a body length of 10 – 13 cm and a weight of 70 – 100 g. Specimens were fed mainly on snails *(Lymnaea stagnalis* and *Planorbarius corneus*), fish, minced meat and earthworms, but also cooked potatoes and noodles were eaten.

Haemolymph (1 – 1.5 ml per specimen) was obtained from the pericardial sinus by sterile dorsal puncture with a strong hypodermic needle (1 mm in diameter). Haemolymph was immediately mixed in the syringe with the same amount of crayfish-buffered saline according to SÖDERHÄLL et al. (1979), phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM K_2PO_4) or arthropod artificial haemolymph according to WENNING (1989) with or without different amounts of EDTA to inhibit haemolymph clotting and haemocyte disintegration (see below). After puncture the specimens were kept in plastic boxes filled with water that did not reach the dorsal carapace to avoid infections through the wound. The wound from which the haemolymph poured out after puncture was rapidly closed with hot wax; specimens remained in the box for about 1 h and then were put back into the basin. All animals treated this way survived the experiment.

Haemolymph diluted in buffer was carefully dropped on clean microscopic slides and haemocytes were allowed to spread (adhere to the glass slide, flatten and build pseudopodia) for 10 – 15 min at room temperature in a moist chamber. Then they were fixed in 2.5 % glutaraldehyde in aq. dest. for 10 – 15 min at room temperature and the fixative was subsequently removed by rinsing with CFS. Haemocytes were investigated with a Leitz Orthoplan using bright field (BFM) or phase contrast microscopy (PCM).

For Giemsa- (1 : 20 aqueous solution) and May-Grünwald- (1 : 1 aqueous solution) staining fixed haemocytes were covered with the staining solutions for 10 – 15 min. The slides were then rinsed with PBS. The test on intracellular phenoloxidase-activity was made as described by XYLANDER & NEVERMANN (1993): the PO in fixed haemocytes was activated in 30 % ethanol for 15 min and melanisation was enhanced by subsequent incubation of haemocytes with L-dopa or dopamine.

Abbreviations

BFM	Bright field microscopy	MG	May-Grünwald
CFS	Crayfish saline according to SÖDERHÄLL et al. (1979)	P	Presumptive prohaemocyte
		PBS	Phosphate-buffered saline
G	Granular haemocyte	PCM	Phase contrast microscopy
GI	Giemsa	PO	Phenoloxidase
H	Hyaline cells	S	Semi-granular cells

3. Results

Adjusting the buffer system for haemocyte differentiation

Using the original crayfish saline (CFS) by SÖDERHÄLL et al. (1979) the granular and semi-granular haemocytes spread in vitro whereas the hyaline cells completely disintegrate, leaving nothing but naked nuclei. Similar results have also been found using PBS and artificial arthropod haemolymph according to WENNING (1989). With these buffers no differentiation of haemocytes by staining is possible. A fourth cell type (see Fig. 1) was found normally making up less than 0.5 % of the total haemocyte counts; it is considered to be a prohaemocyte but will not be treated here in detail.

Using CFS containing 20 mM EDTA Ca^{2+}-ions are complexed and the hyaline cells no longer disintegrate (Figs 1, 4 & 5). In this solution all types of haemocytes show about the same degree of spreading, leading to a typical spindle shape (Figs. 1, 2, 4 & 5). By flattening in a characteristic way, they exhibit their different sizes and also the volume of nuclei and grana becomes clearly visible (Figs 4 & 6). However, high concentration of Ca^{2+} (0.3 M) also leads to stabilisation of hyaline haemocytes; then all haemocyte types show strong spreading and formation of numerous fine pseudopodia (Fig. 3).

Sizes and spreading characteristics of haemocytes

Hyaline cells are the smallest haemocytes (Figs 4 & 5) with a diameter of 15 µm in buffer with Ca^{2+} and a length of 30 µm in CFS-EDTA; they contain few small or even no grana (Figs 4 & 6). Semi-granular and granular cells both have about the same size in vivo (about 22 µm in diameter) and after spreading in CFS-EDTA (length of the spindle: 45 µm; Figs 4 – 7). The semi-granular cells have small grana (about the size of those of the hyaline cells) and spread intensively in CFS without EDTA (not shown).

In granular cells the spindle shape is less elaborated, most possibly due to the high number of large ovoid grana (2.2 – 4.2 µm in diameter), which may complicate spreading (Figs 4 & 5). Using the spreading characteristics in CFS-EDTA, differentiation of haemocyte types and differential haemocyte counting is rather easy and fast.

Cytochemical staining

After Mai-Grünwald-staining the nuclei appear blue-grey and the plasma light grey in PCM. Grana stain violet in granular haemocytes and light blue in semi-granular and hyaline haemocytes (Figs 1 & 5). Using BFM, all nuclei are moderately grey and plasma light grey (Fig. 4). Grana appear burgundy in granular and brownish-violet in semi-granular and hyaline haemocytes (Fig. 4).

After Giemsa staining nuclei are violet in all haemocytes in BFM (Fig. 6). In granular haemocytes the cytoplasm stains light blue, whereas the cytoplasm of the other cells is violet. Grana are violet-blue in granular cells and light blue in the other types. In PCM plasma is violet in hyaline and semi-granular cells but light blue in granular cells (Fig. 2); nuclei are deeply purple (Fig. 2).

After PO activation by ethanol and L-dopa or dopamine incubation high PO activity (strong melanisation) can be found in the granular cells (Fig. 10) and moderate activity in the semi-granular cells (Fig. 11); since hyaline haemocytes lack this enzyme, they show

no melanisation (Fig. 12). This is easily visible by switching the microscope from PCM to BFM: in BFM only the melanised granular and semi-granular cells are clearly visible, the faint hyaline cells are hardly visible (Figs 8 & 9).

Differential haemocyte counts

Hyaline haemocytes are the most frequent haemocytes in *Astacus leptodactylus* comprising 53.8 to 69.5 % of all haemocytes. Semi-granular haemocytes make up about 24.6 to 29 %, whereas the granular haemocytes comprise only 11.5 to 17.5 %.

4. Discussion

In the literature three main types of haemocytes are described for crustaceans (cf. Bauchau 1981, Hose et al. 1990, Lanz et al. 1993). Only few scientists mentioned further haemocyte types (e. g. Ravindranath 1974). Thus, the haemocytes, at least of the decapod crustaceans on which the investigations have been mainly done, do not show that heterogeneity described for insects and myriapods, where often five to six haemocyte types can be distinguished in a single species (Jones 1962, Price & Ratcliffe 1974, Rowley & Ratcliffe 1981, Nevermann et al. 1991, Xylander 1992). However, one has to take into account that the majority of investigations on crustacean haematograms have been made on adult stages in which haemocytes may have changed showing few »more differentiated« types. Larval stages and their differential haemocyte counts which probably comprise more haemocyte types have not yet been investigated intensively. Furthermore, there is a need for comparative studies on haemocytes of non-decapod crustaceans.

Hyaline cells generally are described to be the smallest haemocytes of crustaceans with a large nucleus-plasma ratio followed by the semi-granular and the granular cells (Bauchau 1981, Hose et al. 1990, Lanz et al. 1993). This coincides with our results.

The function of the haemocytes is not completely known but granular and semi-granular haemocytes contain several components of the phenoloxidase system which are discharged after infections and may lead to the destruction of the pathogen (Johansson & Söderhäll 1985, 1989). Furthermore, Xylander et al. (1997) showed that haemocytes are also involved in the production and storage of at least two different antibacterial substances. Hyaline haemocytes have been shown to be active in phagocytosis (Bauchau 1981, Johansson & Söderhäll 1989), but this also has been reported from the other two main cell types (Hose & Martin 1989, Hose et al. 1990, Baracco et al. 1991). Their capability of rapid disintegration and coagulation of their plasma, however, indicate that they are involved in two different mechanisms of immune defence: nodule formation and wound closure.

As haemocytes obviously are one of the most important parts of internal defence in crustaceans, their rapid and easy differentiation is a valuable tool for further investigations. The methods demonstrated may help to discriminate the haemocytes in crustaceans from other taxa also.

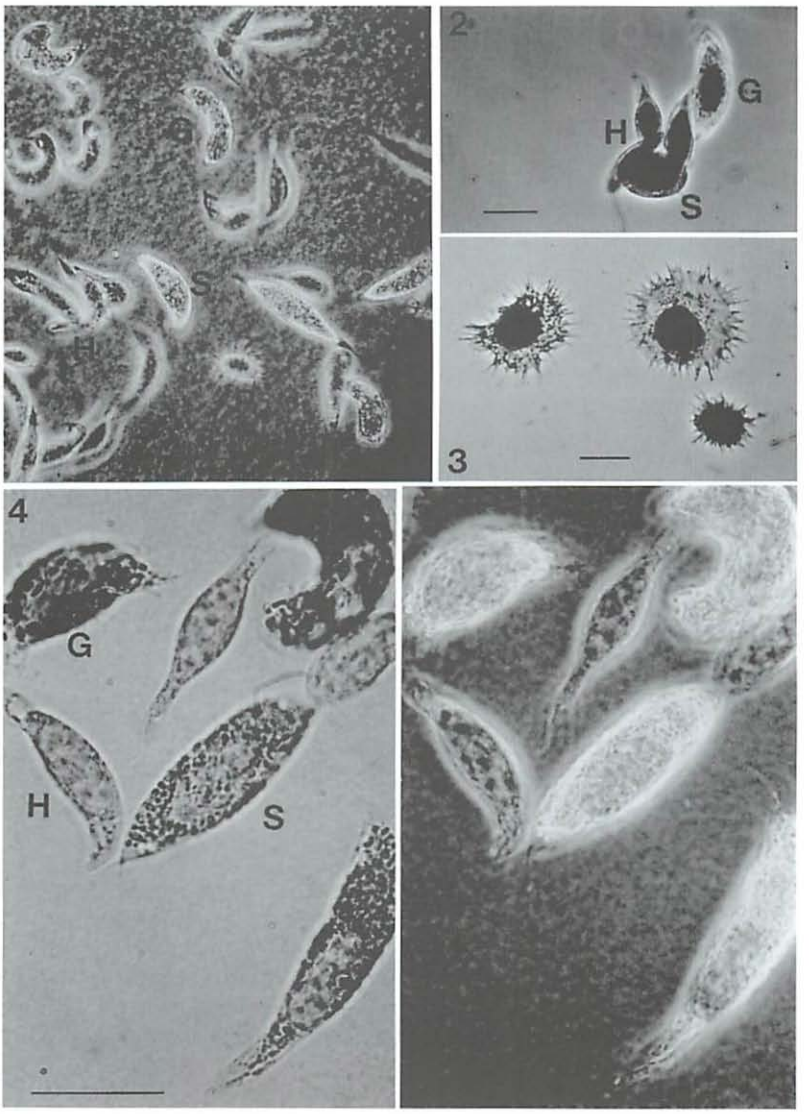

Plate 1

All figures show haemocytes of *Astacus leptodactylus* in CFS with 20mM EDTA if not otherwise indicated

Fig. 1 PCM overview of MG stained haemocytes showing hyaline, semi-granular and granular haemocytes and one of the very rare prohaemocytes. Scale bar: 20 µm

Fig. 2 PCM of GI-stained haemocytes. Scale bar: 15 µm

Fig. 3 PCM of GI-stained haemocytes incubated in CFS containing 0.3 M Ca^{2+} Scale bar: 15 µm

Fig. 4 BFM of MG-stained haemocytes. Scale bar: 25 µm

Fig. 5 Same sector as Fig. 4 in PCM. Scale bar: 25 µm

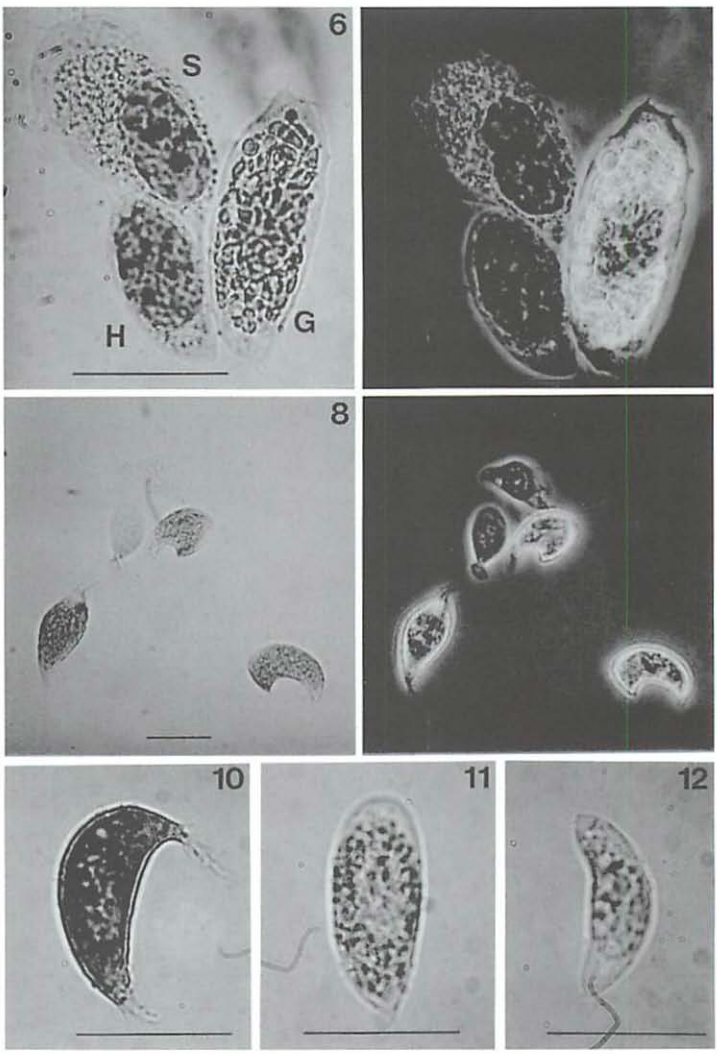

Plate 2

All figures show haemocytes of *Astacus leptodactylus* in CFS with 20mM EDTA if not otherwise indicated

Fig. 6 BFM of different Gl-stained haemocytes (faint staining). Scale bar: 20 µm

Fig. 7 Same sector as Fig. 6 in PCM. Scale bar: 20 µm

Fig. 8 BFM of PO-activated and L-dopa incubated haemocytes. Scale bar:20 µm

Fig. 9 Same sector as Fig. 8 in PCM. Scale bar: 20 µm

Fig. 10 PO-activated granular haemocyte after dopamine incubation (BFM). Scale bar: 25 µm

Fig. 11 PO-activated semi-granular haemocyte after dopamine incubation (BFM). Scale bar: 25 µm

Fig. 12 Hyaline haemocyte after corresponding treatment as Figs 10 & 11 showing no PO-activity (BFM). Scale bar: 25 µm

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