Morphology of Hemocytes From the Freshwater Prawn
Macrophium rosenbergii

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ABSTRACT Using morphological criteria, we identified three types of blood cells in the freshwater prawn Macrophium rosenbergii. Hyaline hemocytes, the most abundant type, have few large cytoplasmic granules, a large nucleocytoplasmic ratio, and lyse spontaneously in the absence of anticoagulant. Granular hemocytes are heterogeneous in size and in density of their granules. They are phagocytic and readily spread on substrates. The third type of hemocytes, identified as undifferentiated hemocytes, are the least abundant. The hemocytes of this economically relevant crustacean are compared with blood cells of other decapods. J. Morphol. 234:147–153, 1997.

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Blood cells and seric factors in arthropods play important roles in defense mechanisms against parasites and pathogenic or nonpathogenic microorganisms that might enter the hemocoel through wounds in the cuticle (Ravindranath, '80; Ratcliffe et al., '84). In decapod crustaceans and insects, circulating hemocytes appear to be critical to defense (Johansson and Söderhäll, '89; Martin and Hose, '92). In vitro and in vivo studies have shown the participation of hemocytes in defense mechanisms such as phagocytic activity (Tyson and Jenkin, '74; Smith and Ratcliffe, '78), the prophenoloxidase activating system, induction of degranulation of hemocytes by endogenous proteins (Ratcliffe et al., '84; Söderhäll et al., '84; Johansson and Söderhäll, '89) and cellular encapsulation and nodulation (Ratner and Vinson, '83; Persson et al., '87). Morphological studies of the cells from crustacean hemolymph have shown the presence of three types of circulating hemocytes: hyaline, small-granule, and large-granule cells (Mix and Sparks, '80; Martin and Graves, '85; Hose et al., '87; Tsing et al., '89). The main differences used in the classification of hemocytes are the presence of cytoplasmic granules and the proportion of granules in hyaline and granular cells (Mix and Sparks, '80; Martin and Graves, '85; Hose et al., '87; Tsing et al., '89). However, hemocyte morphology shows enough variation that any classification scheme based on morphology should be backed up by studies on the function and cytochemistry of the hemocytes (Martin and Hose, '92).

The purpose of this report is to describe the morphology of hemocytes from an important freshwater decapod, Macrophium rosenbergii, and to compare these cells to those of the more thoroughly studied marine species.

MATERIAL AND METHODS

Animals

Males of freshwater prawns, Macrobrachium rosenbergii (DeMan), were obtained

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from the aquaculture farm "El Huamuchil," Jojutla, Morelos, Mexico, and kept in aerated tanks in the laboratory. Animals in molt stage C (weighing 15–20 g) were identified according to the characteristics described by Peebles ('77).

**Examination of hemocytes**

Since crustacean hemocytes change shape or lyse almost spontaneously following removal from the body, the hemolymph was withdrawn from the pericardial sinus using a 1.5”, 21-gauge needle on a syringe containing anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid and 20 mM EDTA, pH 4.5) and a fixative solution (consisting of 1.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.8) (Söderhäll and Smith, '83). The anticoagulant and fixative solutions were mixed 1:1 v/v and cooled to 4°C, and the hemolymph was collected in a 9:1 proportion (anticoagulant-fixative: hemolymph). Cells in suspension were pelleted by centrifugation (360 × g for 5 min, 4°C), then suspended in fixative for 2 hr at 4°C, and finally washed two times in 0.1 M sodium cacodylate buffer, pH 7.8. These samples were used to determine differential hemocyte counts using the 10× objective of a light microscope (Olympus BH-2). The average length and width was measured with a ruler with a 1:100 scale using the 100× objective. Morphological observations were made with differential-interference-contrast (DIC) microscopy (Nomarski) using a Zeiss photomicroscope.

**Biological activity of hemocytes**

To examine cellular viability, adherence capacity, and phagocytic activity, hemolymph was obtained in anticoagulant solution (1:6 v/v). The total number of hemocytes was counted with a hemocytometer and then the cellular viability was tested by the trypan blue-dye-exclusion method (>90% viability). In order to test the adherent capability of hemocytes, the cells were washed by centrifugation (360 × g for 5 min, 4°C) and incubated for 30 min at room temperature (22 ± 2°C) in tissue plates (Nunc, Denmark) in Van Harrevald’s ('36) solution (VHS): 0.2 M NaCl, 0.1 M CaCl₂, 2 mM MgCl₂, 5 mM KCl, 2 mM NaHCO₃, pH 7.4. Nonadhered hemocytes were eliminated by washing with VHS and the identity and percentage of adherent hemocytes were determined. The adhered hemocytes were further tested for their phagocytic abilities. Hemocytes were incubated in the presence of chicken erythrocytes (previously washed in 0.9% NaCl solution) at a ratio of 10:1 erythrocyte/hemocyte for 30 min at 25°C. Phagocytic hemocytes were identified by phase-contrast microscopy. Non-phagocytized erythrocytes were removed by washing with VHS. Some preparations were fixed with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.8, for 2 hr at 4°C, stained with Harris’ hematoxylin (1 min) and eosin (4 sec) (H&E), and examined by Nomarski DIC microscopy using a Zeiss photomicroscope.

**Cell preparation for electron microscopy**

For transmission electron microscopy (TEM), hemocytes in suspension, fixed as described above, were postfixed in 1% OsO₄ in 0.1 M sodium cacodylate buffer, dehydrated in gradually increasing concentrations of ethanol, transferred to propylene oxide, and embedded in Araldite 6005. Thin sections were stained with Reynolds ('63) lead citrate and observed in a Zeiss EM 109. Samples of adherent cells to be examined by scanning electron microscopy (SEM) were fixed as described above for TEM, dried to critical point, and observed in a DSM 950 SEM.

**RESULTS**

**Hemocyte classification**

Hemocytes from *M. rosenbergii* show morphological differences from hemocytes of other decapods. However, in order to avoid complications in the classification of *M. rosenbergii* cells, we apply standard terminology and note differences in the discussion. Morphologically, three main types of hemocytes are observed in the freshwater prawn: hyaline hemocytes, granular hemocytes, and undifferentiated hemocytes (Fig. 1).

**Hyaline hemocytes**

This group of cells comprises 70% of the circulating hemocytes. They are readily identified by their fusiform or spindle shape (31 μm × 5 μm length and width, respectively). In most cells, the elongated nucleus is located centrally, but other cells show an eccentric location and therefore have only one tapered end (Fig. 1). The typical fusiform hyaline hemocytes are easily identified under TEM (Fig. 2). Each has an elongated and irregularly outlined nucleus, with abundant
nuclear envelope-associated heterochromatin and prominent perinuclear cisternae. The cytoplasm is characterized by the presence of spherical electron-dense granules and less abundant rod-shaped granules (Fig. 2, insert). Many irregular shape cytoplasmic areas with finely granular and weakly electron-dense contents are observed close to the granules. Other cytoplasmic organelles are poorly developed, but rough endoplasmic reticulum (RER) is observed as long and widespread cisternae, particularly in hyaline hemocytes with one tapered end and few cytoplasmic granules (Fig. 3, insert). These cells could be the immature form of fusiform hyaline hemocytes. Those hyaline hemocytes, observed by SEM, in adhered-cell preparations show the characteristic fusiform shape, with some short filopodia and pseudopod-like processes attached to the surface of the tissue culture chambers (see Fig. 7a).

**Granular hemocytes**

These hemocytes (25 µm × 9 µm) comprise 20% of suspended cells and contain numerous cytoplasmic granules (Fig. 1). The granulocytes observed by TEM show variation in size and density of their cytoplasmic granules; hence we consider that these cells may be divided into two groups. Type I granulocytes (Fig. 4) have a high nucleocytoplasmic ratio and contain irregular shape electron-dense cytoplasmic granules associated with electron-lucent cytoplasmic areas, as described for hyaline hemocytes. Some
Fig. 4. Small granular hemocytes of *Macrobrachium rosenbergii* fixed immediately after removal from circulation exhibit irregular shape granules. Some granules show inner areas of increased electron density (arrowheads) or an inner linear array (inserts). There is a close association between granules and electron-lucent cytoplasmic areas (inserts). TEM.

Fig. 5. Larger granular hemocytes of *Macrobrachium rosenbergii* fixed immediately after removal from circulation possess the same class of granules as depicted in Figure 4, but the granules with intermediate electron density are more numerous (arrows). Very electron-dense granules are pleomorphic (arrowheads). Both classes of granules are associated with electron-lucent cytoplasmic areas (inserts). The endoplasmic reticulum is well developed and the nucleus is frequently elongated and indented. TEM.

granules show a parallel linear substructure (Fig. 4, inserts). RER is present, but other organelles in our preparations appear to be absent. Type II granulocytes (Fig. 5) have more numerous granules that almost fill the cytoplasm. In addition, pleomorphic and very electron-dense granules are present in these cells and the granules and areas of cytoplasm containing electron-lucent vesicles are closely associated (Fig. 5, inserts). Adhered-cell preparations, as observed by SEM, show that granular hemocytes are characterized by extensive spreading and formation of pseudopodia and long filipodia (see Fig. 7b,c).

**Undifferentiated hemocytes**

This group comprises 10% of circulating hemocytes and are disc-shaped when observed by DIC (Fig. 1). These smallest hemocytes (13.7 ± 5 μm × 7.5 ± 2 μm) have a high nucleocytoplasmic ratio and contain no or very few cytoplasmic granules (Fig. 6). Nuclei of undifferentiated hemocytes are centrally placed with perinuclear cisternae and abundant nuclear envelope-associated heterochromatin. A poorly developed RER is the only organelle observed. With SEM, we observed some smaller adhered cells with a few, short pseudopodia and filipodia. These cells probably correspond to undifferentiated hemocytes (Fig. 7d).

**Biological activities**

**Hyaline hemocytes**

After hemocytes were allowed to settle onto tissue plates and washed with VHS, the differential counts show that hyaline hemocytes made up only 30% of the attached cells.

Fig. 6. Undifferentiated hemocytes of *Macrobrachium rosenbergii*, fixed immediately after removal from circulation, are characterized by the scarcity or absence of cytoplasmic granules and the high nucleocytoplasmic ratio. TEM.
the rate of incorporation of trypan blue is not increased after adherence to the tissue-culture chambers.

**DISCUSSION**

The hemocytes of *M. rosenbergii* do not fit easily into classification schemes recently presented for marine decapods. In many reports, hemocytes from crustaceans are grouped into two types: hyaline and granular hemocytes (Martin and Hose, '92). In this work, we identify three types of circulating hemocytes from the freshwater prawn *M. rosenbergii*, a species that has worldwide economic importance in aquaculture. The first type of cells is the hyaline hemocyte, which represents the most abundant group of blood cells, has a fusiform shape, and contains few electron-dense cytoplasmic granules and large, irregular shape, electronlucent cytoplasmic areas. These fusiform hyaline hemocytes stain with trypan blue and have a great tendency to form clusters and to lyse. They seem to correspond to the hyaline cells identified by Hose et al. ('90); in other species of decapods, such as the spiny lobster *Panulirus interruptus* and the ridgeback prawn *Sicyonia ingentis*. In these organisms, the hyaline cells are basically agranular and are the most abundant hemocytes, comprising 61% of the circulating cells, just as in *M. rosenbergii*. In the American lobster *Homarus americanus*, this type of cells represents 22% of the circulating hemo-

Although this group of cells adheres to the culture plates (Fig. 7a), they show a high tendency to lyse, determined not only by the differential counts, but also by their positive staining with trypan blue and tendency to form clusters. Preliminary observations indicate that cytolysis occurs in approximately 60 sec; however, in the presence of anticoagulant solution, cytolytic activity is not observed until after 30 min.

**Granular hemocytes**

This group of hemocytes, adheres to the plastic culture chamber by spreading and flattening and shows numerous filopodia and pseudopod (Fig. 7b,c). Some cells that express a more prominent formation of filopodia and pseudopodia probably correspond to the type II granulocytes (Fig. 7c); moreover, they are the only cells that phagocytize chicken erythrocytes (Fig. 8).

**Undifferentiated cells**

When allowed to adhere to tissue culture chambers, these cells show several short pseudopodia, but they do not show phagocytic activity with chicken erythrocytes. Very few of these cells stain with trypan blue and

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**Fig. 7.** *Macrobrachium rosenbergii* hemocytes fixed after 30 min of adhesion on tissue plates. (a) Hyaline hemocytes conserve their fusiform shape and produce few and short pseudopodia. (b,c) Granular hemocytes undergo profound morphological changes characterized by a broad-spreading pseudopodia and filopodia formation. (d) Some cells with intermediate spreading aspect but with a clear adhesion behavior probably correspond to undifferentiated cells. SEM.

**Fig. 8.** Granular hemocytes of *Macrobrachium rosenbergii* after phagocytosis assay. Granulocytes are the only cells with phagocytic activity and show morphological features similar to the cells of Figure 7b,c. n, nucleus; e, phagocytized erythrocytes. Hematoxylin and eosin. ×1260. Nomarski DIC.
cytes but has numerous small granules (Hose et al., '90). In some decapods, such as S. ingentis, Omori et al. ('89) demonstrated that hyaline cells possess lytic and clotting activity. In M. rosenbergii, hyaline fusiform hemocytes seem to possess autolytic activity as well, as suggested by their high capacity to take up trypan blue, to lyse spontaneously, and to form clots.

The granulocytes make up 20% of the hemocytes in M. rosenbergii. They are large, ovoid cells characterized by the presence of electron-dense granules. As observed by several authors, crustacean hemocytes contain granules whose size and number vary greatly. For this reason, granulocytes have often been subdivided into two types: small- and large-granule hemocytes. The small-granule hemocytes (also termed semigranulocytes) comprise 29% of the total cells in P. interruptus, 60% in H. americanus, and 18% in Penaeus japonicus (Tsing et al., '89; Hose et al., '90). These cells show a low nucleocytoplasmic ratio and many cytoplasmic granules, which are typically spherical in shape. The large granular hemocytes, also defined as refractile cells by Hose et al. ('90), are recognized by the presence of large granules that fill the cytoplasm. This cellular type comprises 9.8% of the total hemocytes in P. interruptus, 16% in H. americanus, and, as reported by Tsing et al. ('89), 12% in P. japonicus.

The granulocytes in M. rosenbergii can be divided into two types, but neither one corresponds to the large, refractile variety found in other species. Type I granulocytes in M. rosenbergii are smaller and contain fewer granules than type II. In addition, type II granulocytes contain pleomorphic, very electron-dense granules and are responsible for the majority of phagocytic activity as has been described for granulocytes in other crustaceans (see Martin and Hose, '92).

The third type of hemocytes identified in freshwater prawns makes up 8% of the circulating blood cells. They are small, rounded cells, almost devoid of cytoplasmic organelles and granules and probably represent an undifferentiated group of cells. The term "undifferentiated hemocytes" has been applied by Tsing et al. ('89), for those cells in P. japonicus that possess dispersed nuclear chromatin, moderately developed RER, and sometimes scarce, small, rounded cytoplasmic granules. The third hemocyte type in M. rosenbergii possesses ultrastructural fea-

tures similar to those of P. japonicus; however, they have an adherent behavior but no phagocytic activity. If adhesion to the substrate is a feature of phagocytic cells, the undifferentiated hemocyte of M. rosenbergii could represent the more immature form of granular hemocytes.

To summarize, M. rosenbergii, like other decapods, has fusiform hyaline cells that are probably involved in clotting the hemolymph and granulocytes that phagocytize foreign materials. Macrobrachium rosenbergii lacks the large, refractile-granule hemocytes present in other species, but it possesses a third group of small, possibly undifferentiated, hemocytes in circulation.

In future work we plan to apply more specific functional tests and phenotypic markers to the characterization of these cells. An understanding of the circulating hemocytes in M. rosenbergii may allow for future studies to assess the physiological status of individuals exposed to stress, injury, or infection by examining specific hemolymph parameters.

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LITERATURE CITED


