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Morphological Characterization via Light and Electron Microscopy of the Hemocytes of two Cultured Bivalves: A Comparison Study between the Hard Clam (*Meretrix lusoria*) and Pacific Oyster (*Crassostrea gigas*)

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Su-Jung Chang, Su-Min Tseng, and Hsin-Yiu Chou (2005) Morphological characterization via light and electron microscopy of the hemocytes of two cultured bivalves: a comparison study between the hard clam (*Meretrix lusoria*) and Pacific oyster (*Crassostrea gigas*). *Zoological Studies* **44**(1): 144-153. In this study, the circulating hemocytes of the hard clam (*Meretrix lusoria*) were characterized by light and electron microscopic observations and simultaneously compared with those of the Pacific oyster (*Crassostrea gigas*). First, two cell types, granulocytes and agranulocytes, were identified based on the existence of cytoplasmic granules under light microscopy. The hemocytes were then stained and the granulocytes subclassifed into eosinophilic and basophilic granulocytes. In the oyster, three types of granulocytes were observed: eosinophilic, basophilic and an intermix. Conversely, the main type of granulocyte in the hard clam was eosinophilic granulocytes, and distinctive small and large granules were recognized. Agranulocytes in the hard clam could be subdivided into hyalinocytes and blast-like cells. Another cell type, vesicular cells, was observed in the oyster as unclassified cells. To further characterize hemocyte populations in both species, transmission electron microscopic observations were carried out. In hard clam granulocytes, abundant electron-dense cytoplasmic granules of two distinctive sizes were observed as recognized in light microscopy. However, two granulocyte types with either electron-dense or electron-lucent granules were found in the oyster, with the latter likely being basophilic granulocytes, which were seldom found in the hard clam. In addition, hemocytes with few or no cytoplasmic granules, and possessing few organelles such as mitochondria, Golgi complexes, and endoplasmic reticula, were found in the agranulocytes of both species. Blast-like cells, however, were particular in the hard clam in that they were small and had a high nucleus: cytoplasm ratio, while lacking most organelles, except mitochondria.
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Key words: Morphology, Hemocyte, Hard clam, Oyster.

The culturing of the hard clam (*Meretrix lusoria*) and Pacific oyster (*Crassostrea gigas*) constitutes some of the most economically important fisheries along the southwestern coast of Taiwan. However since 1969, hard clams have suffered a high degree of mortality each spring and/or summer (Tseng 1976). Several factors have been implicated as possible causes of this mass mortality, such as variations in temperature and salinity, industrial and pesticide pollution, and infectious diseases (Tseng 1976, Yang et al. 1978, Kou et al. 1989). The annual production of oysters and oyster larval settlement has also seriously diminished in recent years for undetermined reasons. This situation, involving both clams and oysters, continues, although the exact cause still remains unclear. Since bivalve hemocytes play an important role in homeostatic functions and defense mechanisms, the morphological characterization of hemocytes is a prerequisite to further exploring the causes of death in these two bivalves (Cheng 1981, Fisher 1986).

In general, two basic cell types are recognized among bivalve hemocytes: agranulocytes

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and granulocytes, which are dependent on the presence of cytoplasmic granules (Cheng 1981, Auffret 1988). Three types of agranulocytes have been identified: blast-like cells, basophilic macrophage-like cells, and hyalinocytes (Hine 1999). On the other hand, granulocytes may be subdivided into neutrophils, acidophils and basophils (Cheng 1981, McCormick-Ray and Howard 1991, López et al. 1997). Nevertheless, different opinions concerning these classifications of hemocytes in bivalves exist due to different techniques used. For example, although the hemocytes of the Pacific oyster have been studied for a long period of time, types of subpopulations and their morphofunctions are still controversial (Ruddell 1971a b c, Feng et al. 1977, Bachère et al. 1988, Auffert 1989).

M. lusoria is a species of shellfish species cultured in Taiwan, and only very little information is available on *M. lusoria* hemocytes (Wen et al. 1994, Park et al. 2002). The hemogram, i.e., the number and constitutes of hemocytes, is believed to be an essential parameter for characterizing the immune capacity in bivalves. To further investigate details of the defense mechanism and the influence of the environment on the hemogram of the hard clam, a more-precise characterization of hard clam hemocytes is needed. Hence, morphological characterization and subtyping of hard clam hemocytes were established and compared with oyster hemocytes via light and electron microscopy in the present study.

MATERIALS AND METHODS

Experimental animals

The experimental animals (*M. lusoria* and *C. gigas*) used in this study were obtained from the Taihsi Station, Marine Aquaculture Research Center, Fisheries Research Institute (FRI) in central Taiwan. Before the experiments took place, all of the animals were kept in aerated tanks for 3 to 7 d, where water temperature and salinity were maintained at 25 ± 1°C and 33 ppt, respectively.

Hemolymph collection

Hemolymph was collected from the posterior adductor muscle sinus of both the clam and oyster, using a sterile syringe with a 25-gauge needle.

Observation of live hemocytes

The collected hemolymph was placed into 6-well plates (Costar) and permitted to settle at room temperature for 10~20 min. Live hemocytes attached themselves to the surface, therefore non-adherent cells were carefully washed off with sterile seawater. The adhering hemocytes were further examined under an inverted microscope with phase-contrast optics (Olympus IMT-2) and then photographed.

Light microscopy

Hemolymph from the experimental animals was placed onto glass slides, and cells were allowed to settle for 10~20 min at room temperature. After light washing, the adherent cells were fixed with 1% glutaraldehyde in artificial seawater for 1 h at 4°C. The slides were then carefully washed with water and stained with May-Grünwald Giemsa stain. Following this, observations were carried out with an Olympus BH-2 light microscope, and differential hemocyte counts were evaluated after counting 100 stained cells.

Transmission electron microscopy (TEM)

Hemolymph from either 5 clams or 5 oysters was pooled for this assay. The collected hemolymph was immediately mixed with an equal volume of 5% glutaraldehyde fixative solution (5% glutaraldehyde in 0.1 M cacodylate buffer; pH 7.3, 1030 mOsm) and centrifuged at 600 xg for 15 min. After removing the supernatant, the cell pellets were again prefixed with 2.5% glutaraldehyde fixative solution for 1.5 h at 4°C. Following this, pellets were washed several times with cold cacodylate buffer, and then postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at 4°C. Samples were dehydrated in a graded series of ethanol and absolute acetone, before being embedded in Spurr's resin. Ultrathin sections were prepared on a Richert-Jung Ultracut E Ultratome, and stained with uranyl acetate and lead citrate. Finally, these sections were observed with a Hitachi H-7100 transmission electron microscope.

RESULTS

Observation of live hemocytes

Live hemocytes of both the hard clam and oyster spontaneously adhered to form pseudopods

and began migration when placed on the culture plates (Fig. 1). First, the 2 main cell types in both bivalves, granulocytes and agranulocytes, were distinguished according to the existence of cytoplasmic granules. The granulocytes frequently showed an eccentric nucleus and contained various granules in the cytoplasm (Fig. 1a, b, d). They also slowly spread and were highly mobile after spreading. Agranulocytes had clear cytoplasm and produced long pseudopods (Fig. 1c, e), which spread rapidly but showed weak mobility. Other cell types were also occasionally observed in *C. gigas*; they had no granules, but lucent vacuoles, which are called vesicular cells (Fig. 1f). Vesicular cells were never found in the hard clam.

Light microscopy

In the stained hemocyte monolayers from both bivalves, granulocytes were further subclassifed into eosinophilic and basophilic granulocytes (Fig. 2). In *M. lusoria*, only eosinophilic granulocytes were observed; moreover, two distinctive sizes of granules were recognized (Fig. 2a, b). Small eosinophilic granulocytes contained many small granules which stained light pink and were found to be the most numerous (64%). The large eosinophilic granulocytes had larger deeply pink-stained granules and comprised 3% of the hemocytes. Conversely, in *C. gigas*, granulocytes had prominent lucent ectoplasm with numerous thin

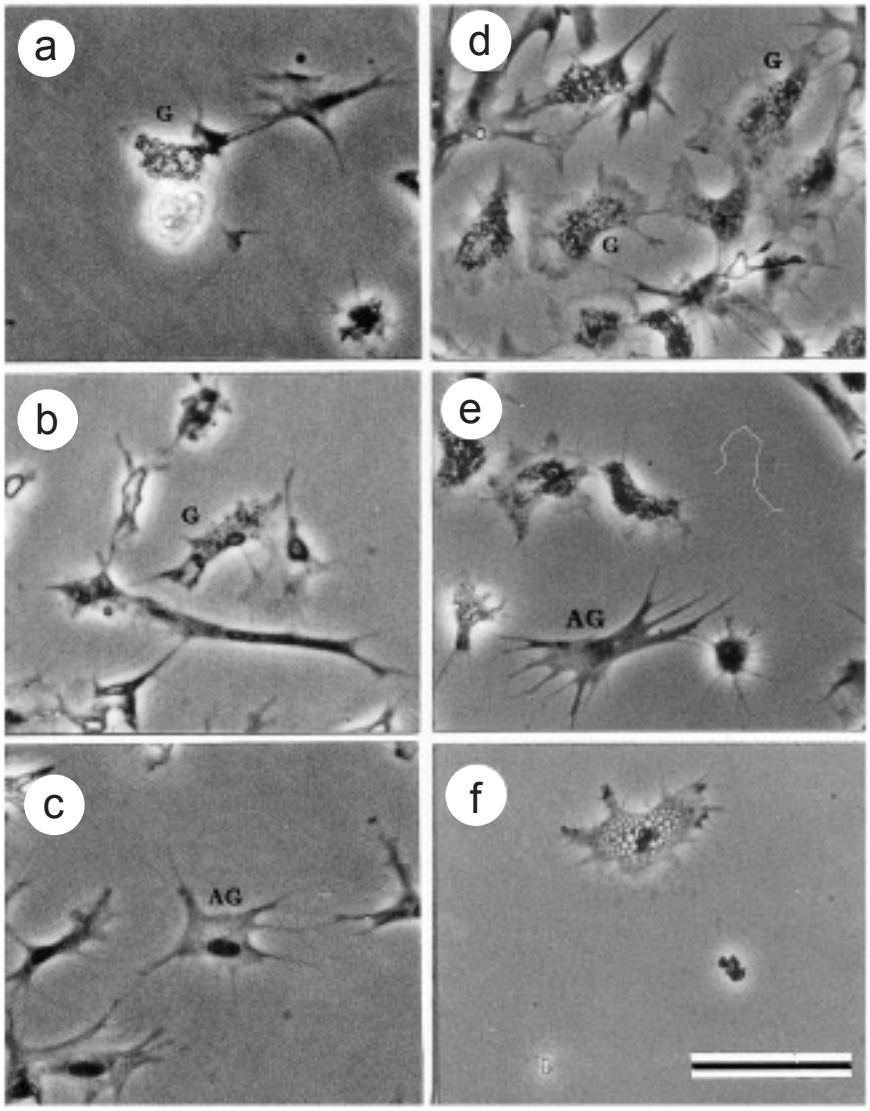

Fig. 1. Live hemocytes viewed with phase-contrast optics. a-c: *Meretrix lusoria*. (a, b) Granulocytes (G); (c) agranulocyte (AG). d-f: *Crassostrea gigas*. (d) Granulocytes (G); (e) agranulocyte (AG); (f) vesicular cell. Scale bars = 50 μm.

filopodia, and three types of granules, eosinophilic, basophilic, and an intermix were observed (Fig. 2e-g). The percentages of these three granulocytes of the total hemocyte count were 25% for basophilic granulocytes and 11% for eosinophilic granulocytes, while cells with a mixture of both granules were rare.

Agranulocytes in *M. lusoria*, generally devoid of granulations, were divided into hyalinocytes (28%) and blast-like cells (5%) (Fig. 2c, d). Hyalinocytes were larger with slightly basophilic cytoplasm, and some contained vacuoles. Flattened hyalinocytes, spread by extending pseudopodia, were observed in the smears. Blast-like cells were relatively smaller, round, and basophilic in appearance and possessed a high

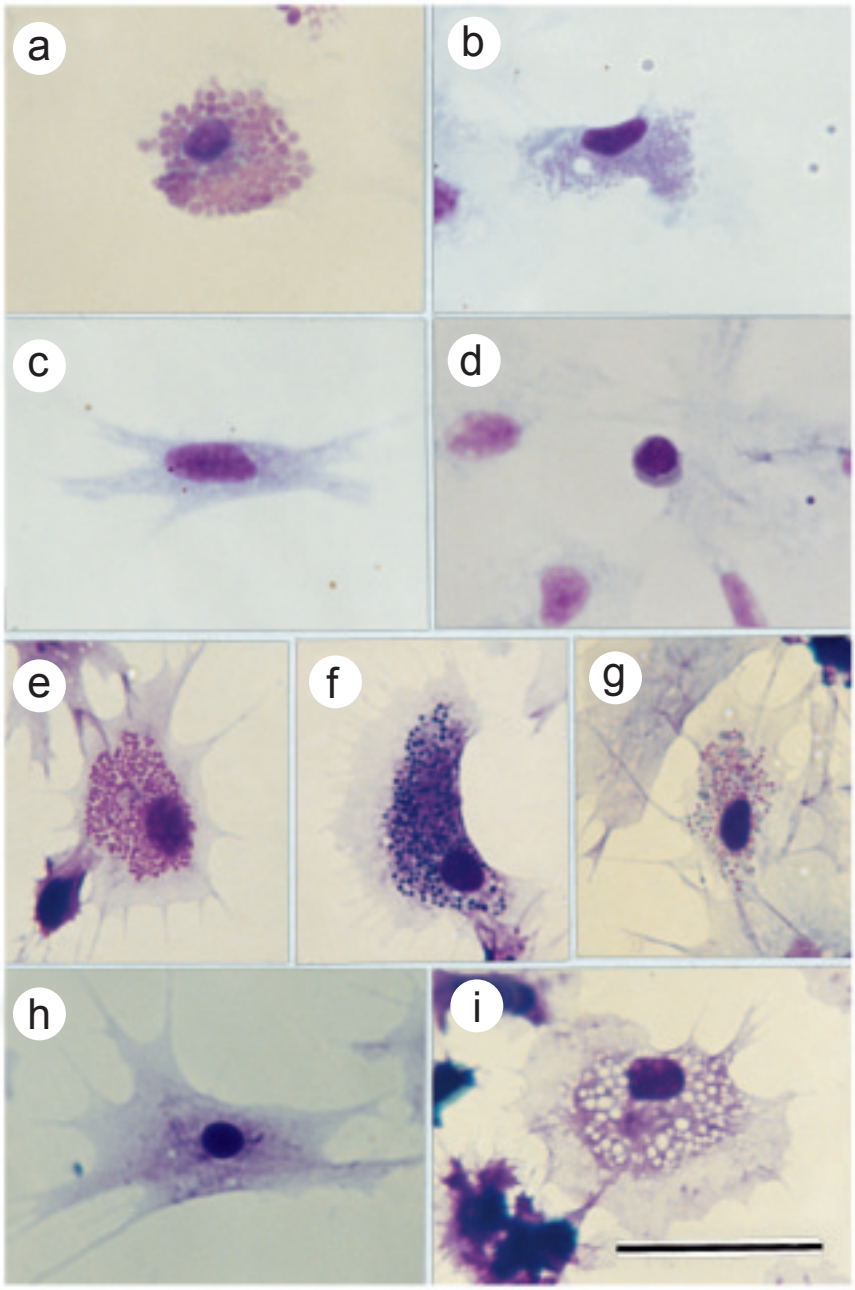

Fig. 2. Light micrographs of hemocytes stained with May-Grünwald Giemsa stain. a-d: *Meretrix lusoria*. (a) Large eosinophilic granulocyte; (b) small eosinophilic granulocyte; (c) hyalinocyte; (d) blast-like cell. e-i: *Crassostrea gigas*. (e) Eosinophilic granulocyte; (f) basophilic granulocyte; (g) granulocyte with intermix of eosinophilic and basophilic granules; (h) agranulocyte; (i) vesicular cell. Scale bars = 20 μm.

nucleus: cytoplasm ratio. In *C. gigas*, agranulocytes with slightly basophilic cytoplasm and long pseudopodia were similar to the hyalinocytes in *M. lusoria* (Fig. 2h). The most-numerous hemocytes in *C. gigas* were the agranulocytes, which accounted for 64%. Another cell type, vesicular cells, was observed and has previously not been classified (Fig. 2i). These cells had eccentric nuclei and prominent ectoplasm, similar to those of the granulocytes, but they had vacuolated cytoplasm and lacked cytoplasmic granules, which made these cells distinctive.

Transmission electron microscopy (TEM)

Common characteristics of both hard clam and oyster granulocytes were observed under TEM (Fig. 3). They all possessed an eccentric nucleus with large clumps of chromatin, a few thin cytoplasmic projections, and a low nucleus: cytoplasm ratio. Variable numbers of mitochondria, Golgi complexes, vesicles and cytoplasmic granules were simultaneously contained in the cytoplasm. Differently, two oyster granulocyte types, having either electron-dense or electron-lucent

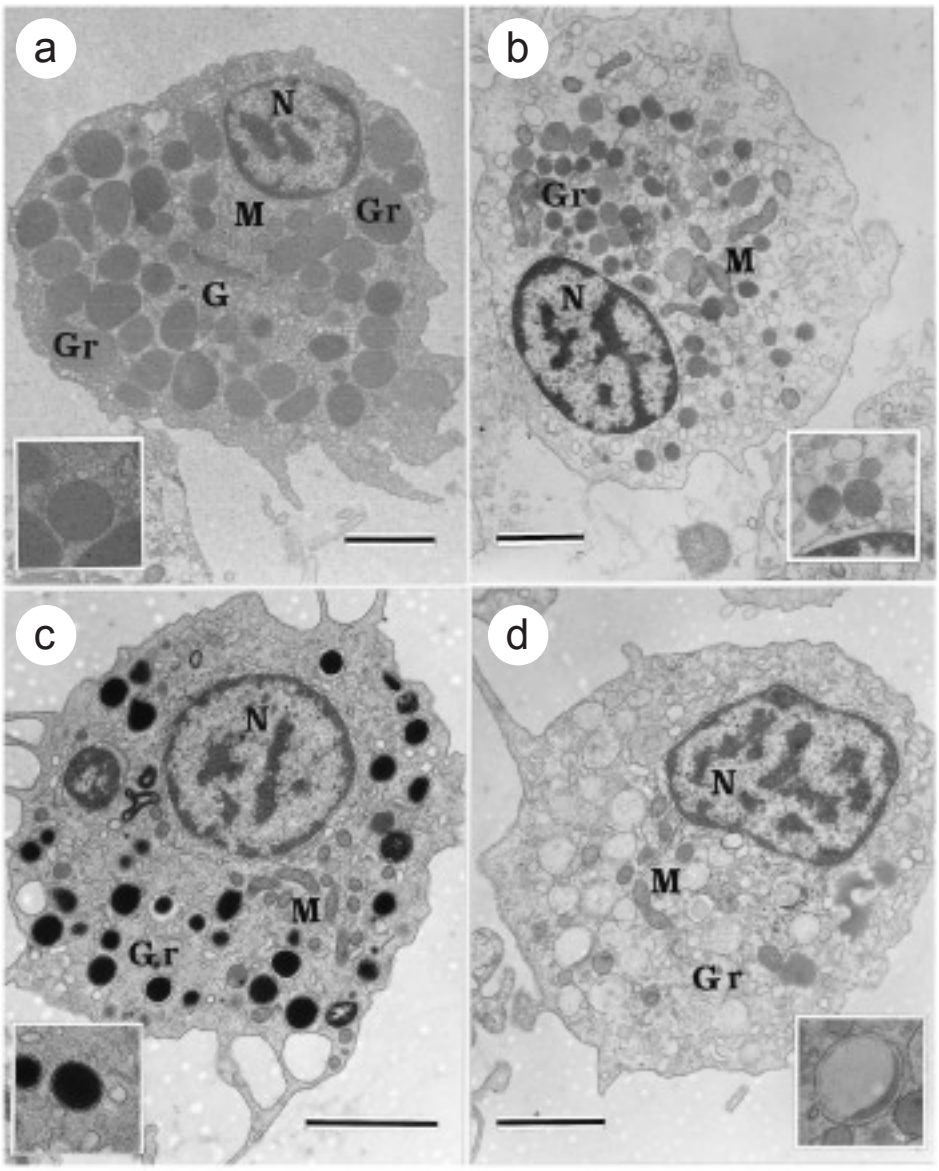

Fig. 3. Transmission electron micrographs of granulocytes. a, b: *Meretrix lusoria*. (a) Granulocyte containing large electron-dense granules; (b) granulocyte containing small electron-dense granules. Inset: high magnification of granules (12,000x). c, d: *Crassostrea gigas*. (c) Granulocyte containing electron-dense granules; (d) granulocyte containing electron-lucent granules. Inset: high magnification of granules (15,000x). N, nucleus; G, Golgi complex; Gr, granule; M, mitochondria. Scale bars = 2 μm.

granules, were found (Fig. 3c, d). The electron-dense and electron-lucent granules were both membrane-bound and spherical, and measured 0.3~0.6 and 0.4~0.9 μm in diameter, respectively. The electron-dense granules were composed of a homogenous electron-dense matrix, while the electron-lucent granules, with an electron-lucent core, were enclosed by an electron-dense material along the membrane (Fig. 3c, d insets). However, in hard clam granulocytes, only electron-dense cytoplasmic granules that were membrane-bound, round, ovoid or irregularly shaped were observed (Fig. 3a, b insets). Furthermore, granulocytes with 2 distinctive sizes of granules, ranging from either 0.5~2 or 0.2~0.8 μm, were recognized with light microscopy. These electron-lucent granules were seldom found in *M. lusoria*.

The hyalinocytes of both species tested had a nucleus and various organelles similar to granulocytes, but lacked cytoplasmic granules (Fig. 4). A nucleus with stippled chromatin, surrounded by long profiles of rough endoplasmic reticula, was observed in both hard clam and oyster hyalinocytes. On the other hand, the nucleus of *M. lusoria* hyalinocytes was larger and ovoid and either reniform or irregularly shaped (Fig. 4a). Blast-like cells of the hard clam were small and had a central ovoid or spherical nucleus surrounded by a rim of scant cytoplasm lacking most organelles, except mitochondria (Fig. 4b). *C. gigas* hyalinocytes had more-prominent pseudopods, and residual bodies were occasionally detected in the cytoplasm (Fig. 4c). Blast-like cells were never seen in *C. gigas*.

A summation of results concerning the morphological characteristics of the principal hemocyte types in *M. lusoria* and *C. gigas* is given in table 1.

DISCUSSION

Morphological criteria were considered generally in order to characterize hemocytes in bivalves, however, the existing nomenclature of bivalve hemocytes is inconsistent, being dependent on the observer and the technique used (Feng et al. 1971, Ruddell 1971a b, Foley and Cheng 1972, Cheng 1975 1981, Moore and Lowe 1977, Hawkins and Howse 1982, Rasmussen et al. 1985). Cheng (1981) presented a morphological scheme based on numbers of cytoplasmic granules, dividing cells into two types: granulocytes, cells containing granules which rang from very few to numerous; and agranulocytes, cells containing few or no granules. In this study, we identified two main hemocyte types as well in *M. lusoria* and *C. gigas*, under both light and electron microscopy: granulocytes and agranulocytes. In addition, vesicular cells were observed in *C. gigas* as unclassified cells.

In most bivalves, such as the oyster *Crassostrea virginica* (McCormick-Ray and Howard 1991), the mussels *Mytilus edulis* (Noël et al. 1994, Pipe et al. 1997) and *Mytilus galloprovincialis* (Carballal et al. 1997), and the clams *Ruditapes decussatus* (López et al. 1997) and *Tapes philippinarum* (Cima et al. 2000), granulocytes were separated into two subclasses,

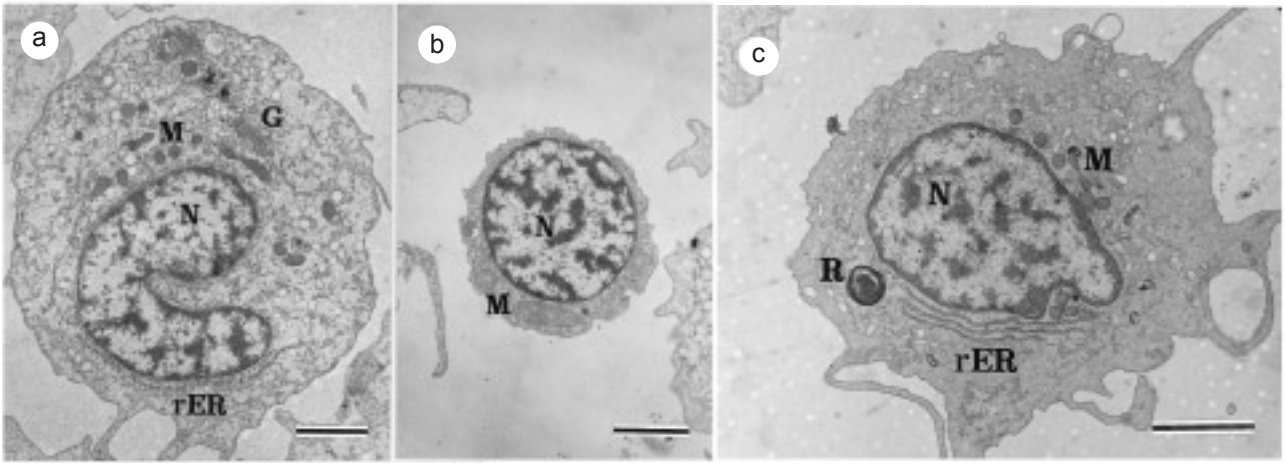

Fig. 4. Transmission electron micrographs of agranulocytes. a, b: *Meretrix lusoria*. (a) Hyalinocyte; (b) blast-like cell. (c) Agranulocyte of *Crassostrea gigas*. N, nucleus; G, Golgi complex; M, mitochondria; rER, rough endoplasmic reticulum; R, residual body. Scale bars = 2 μm.

basophilic and eosinophilic granulocytes, based on their granular affinity to specific dyes. In the stained monolayers, two types of granules were observed in *C. gigas*, while electron-dense and electron-lucent granules were observed in the hemocytes of *C. gigas* by TEM as well. The electron-lucent granules ultrastructurally resembled the granules of other oyster granulocytes and were believed to be basophilic in the stained monolayers (Feng et al. 1971, Bachere et al. 1988, Auffret 1989). These types of granules and granulocytes are believed to be commonly occurring structures in oyster genera (Auffret 1989). Our observations of *C. gigas* confirm a higher percentage of basophilic than of eosinophilic granulocytes. On the other hand, under the same conditions, only one type of granule was observed in *M. lusoria* granulocytes, which was eosinophilic and electron-dense. Although Cheng (1981) suggested that the granules in immature granulocytes may be

Table 1. Morphological characteristics of the principal hemocyte types in Meretrix lusoria and Crassostrea gigas

Meretrix lusoria

Hemocyte type	Granulocyte		Agranulocyte		
Subpopulation	LEG	SEG	H	BLC	EG
Proportion	3%	64%	28%	5%	11%
Nucleus	Eccentric	Eccentric	Central, eccentric	Central	Eccentric
Chromatin	Large chumps	Large chumps	Stripped	Stripped	Large chumps
Cytoplasm					
Ectoplasm	None	Unobvious	None	None	Prominent
Pseudopod	Unobvious	None	Long	None	Prominent
N/C ratio	Low	Low	High	High	Low
Organelles	G, M, V	G, M, V	G, M, V, rER	M	G, M, V
Granules (Gr)	Eosinophilic	Eosinophilic	Lake	Lake	Eosinophilic
Tinctures	Light pink	Deep pink			Deep pink
EM	Electron-dense	Electron-dense			Electron-dense
Size	0.5~2 μm	0.2~0.8 μm			0.3~0.6 μm
Shape	Round, ovoid, rregular	Round			Round, ovoid

Crassostrea gigas

Hemocyte type	Granulocyte		Agranulocyte	Vesicular cell
Subpopulation	BG	Intermix		
Proportion	25%	Rare	64%	Rare
Nucleus	Eccentric	ND	Central, eccentric	ND
Chromatin	Large chumps	ND	Stripped	ND
Cytoplasm				
Ectoplasm	Prominent	Unobvious	None	Prominent
Pseudopod	Prominent	Prominent	Long	Unobvious
N/C ratio	Low	ND	High	ND
Organelles	G, M, V	ND	G, M, V, rER	ND
Granules (Gr)	Basophilic	Eosinophilic and basophilic	Lake	Lake
Tinctures	Deep blue	Pink and blue		
EM	Electron-lucent	ND		
Size	0.4~0.9 μm			
Shape	Round, ovoid			

LEG, large eosinophilic granulocyte; SEG, small eosinophilic granulocyte; H, hyalinocyte; BLC, blast-like cell; EG, eosinophilic granulocyte; BG, basophilic granulocyte; Intermixed, hemocyte with eosinophilic and basophilic granules; Gr, granule; G, Golgi complex; M, mitochondria; V, vesicle; rER, rough endoplasmic reticulum; ND, not detected.

basophilic, becoming acidophilic when mature, basophilic granulocytes have not been observed in some bivalves, such as the clam *Tridacna crocea* (Nakayama et al. 1997) or the mussel *Perna perna* (Barracco et al. 1999). The tinctorial properties of the granules may result from different amounts of hydrolysis enzymes (Moore and Gelder 1983, Gelder and Moore 1986).

Another cell type with lucent vacuoles, named the vesicular cell, was found in *C. gigas*. The cytoplasm of vesicular hemocytes frequently containing endocytotic vacuoles was observed in the stained monolayers. However, since vesicular hemocytes were rarely found, we were unable to observe their morphology by TEM. In view of several morphological similarities, vesicular hemocytes and granulocytes may be related, but these cells have not yet been classified. To date, vesicular hemocytes have only been reported from oysters (Feng et al. 1971, Auffret 1989), cockles (Russell-Pinto et al. 1994), and possibly arcid clams (Holden et al. 1994) and zebra mussels (Giamberini et al. 1996). In our two species, vesicular hemocytes occurred in *C. gigas* but not in *M. lusoria*.

Agranulocytes were divided into two groups in *M. lusoria*: blast-like cells and hyalinocytes. Because the blast-like cells did not easily spread on the slide, observation by phase-contrast microscopy was difficult. Under light and electron microscopic observations, the blast-like cells had typical characteristics of undifferentiated elements, with a narrow rim of basophilic cytoplasm and a high nucleus: cytoplasm ratio. Blast-like agranular hemocytes have been reported from many other bivalve mollusks (Hine 1999), but not all (Nakayama et al. 1997). They are assumed to be hemocyte progenitors based on their basophilic cytoplasm, suggesting the presence of free ribosomes and immaturity (Hine 1999). The same morphological hemocytes were also reported in *T. philippinarum* as hemoblasts. Due to their morphology and positive reaction to the anti-CD34 antibody known to identify hempoietic cells in mammals, these cells are suggested to possibly represent stem cells in bivalves (Cima et al. 2000). In oysters, different observations about agranulocytes have been reported (Bachére et al. 1988, Auffret 1989). Herein, only one agranulocyte cell type, hyalinocytes, was found in *C. gigas*. Some of the hyalinocytes had a higher nucleus: cytoplasm ratio but in contrast to blast-like cells in *M. lusoria*, contained more organelles, such as rER and Golgi complexes. Actually not all hemocyte types occur in each bivalve species. Nomenclature, morphofunctions, as well as differentiation pathways of hemocytes in bivalve species still remain controversial. Inasmuch as *M. lusoria* is a particularly economically important shellfish species cultured in Taiwan, we believe that the morphological characterization of hard clam hemocytes established in the present study can provide further insights into the immune defense of the hard clam, as well as the influences of environmental factors on their hemogram.

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