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Crustacean haemocytes and haematopoiesis

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Abstract

Crustacean haemocytes play important roles in the host immune response including recognition, phagocytosis, melanization, cytotoxicity and cell–cell communication. Classification of the haemocyte types in decapod crustaceans is based mainly on the presence of cytoplasmic granules into hyaline cells, semigranular cells, and granular cells. Each cell type is active in defence reactions, for example; in crayfish, the hyaline cells are chiefly involved in phagocytosis, the semigranular cells are the cells active in encapsulation, while the granular cells participate in storage and release of the prophenoloxidase (proPO) system and cytotoxicity. The haematopoietic tissue has been described in several crustacean decapod species and shown to be the haemocyte-producing organ. Tentative stem cells have been shown to be present in this tissue. Using in situ hybridization, we demonstrated that proPO is not present in the haematopoietic tissue of crayfish which suggests that protein expression is different between circulating haemocytes and the cells in the haematopoietic tissue.

Keywords: Haemocyte; Haematopoiesis; Crustacean immunity; Invertebrate immunity

1. Introduction

The circulating haemocytes of crustaceans and other invertebrates are essential in immunity, performing functions such as phagocytosis, encapsulation, and lysis of foreign cells (Smith and Söderhäll, 1983a; Ratcliffe et al., 1985; Söderhäll and Smith,

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1986; Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1992). The number of free haemocytes can vary and can, for instance, decrease dramatically during an infection (Persson et al., 1987b; Smith and Söderhäll; 1983a; Smith et al., 1984; Lorenzon et al., 1999). Thus, new haemocytes need to be compensatorily and proportionally produced, and it is commonly believed that haemocytes are released continuously, although at varying rates, from a specialized haematopoietic tissue. This tissue has been identified in several crustacean species (Ghiretti-Magaldi et al., 1977; Hose et al., 1992; Martin et al., 1993; Chaga et al., 1995).

Research in the defensive role of haemocyte in crustacean is rapidly progressing, whereas the knowledge on haematopoietic tissue is limited. In order to elucidate the defence mechanisms and allow comparative studies among the different crustacean species, it would be helpful to establish a uniform classification scheme for crustacean haemocytes and haematopoietic tissue. For most crustacean species, the variation in total haemocyte and differential count values are high between individual animals. Consequently, they cannot be used to evaluate the physiological state of the animal. In this review, we briefly point out some works on crustacean circulating haemocytes, on the morphology of the haematopoietic tissue and the cells found, including molecular characteristics of circulating haemocytes and haematopoietic tissue.

2. Haemocytes

In order to study crustacean haemocytes in an optimal way, it is preferable to work with isolated populations of the different cell types. The success of the isolation of haemocytes depends on the efficiency of anticoagulant used. Söderhäll and Smith (1983) established a protocol for isolation and separation of each cell type for marine decapod species, for example, *Carcinus maenus*, *Cancer pagurus*, *Macropipus depurator*, *Eupagurus bernhardus* and *Nephrops norvegicus* (Söderhäll and Smith, 1983; Smith and Söderhäll, 1991; for review, see Johansson and Söderhäll, 1995). It can be applied to freshwater species, for example, *Astacus astacus* (Smith and Söderhäll, 1983b), and *Pacifastacus leniusculus* (Johansson and Söderhäll, 1985) by changing to a NaCl concentration appropriated for the haemolymph osmolarity of these animals. Pure, viable populations of the cell types are obtained by collection in a low pH citrate–EDTA anticoagulant buffer and separation and isolation by centrifugation on a continuous density gradient of Percoll. In the citrate–EDTA buffer used, citric acid serves to delay cell breakdown while EDTA inhibits prophenoloxidase (proPO) activation and prevents the clotting reaction, which is dependent on Ca^{2+} and transglutaminase (Hall et al., 1999), and this buffer at low pH, in combination with citrate, glucose and NaCl, provides a medium optimal for maintenance of cell integrity without significant loss of cell viability. With some modification, the protocol was used to isolate semigranular cells and granular cells in *Penaeus japonicus* and cell adhesion activity was shown in shrimp (Perazzolo and Barracco, 1997).

Crustaceans have three morphologically different haemocyte types: hyaline, semigranular, and granular cells (Bauchau, 1980). Using different assays, such as phagocytosis, encapsulation, cytotoxicity, haemolysis, cell adhesion, and degranulation (for

review, see Johansson, 1995), the isolated haemocyte populations from several crustacean species, such as the freshwater crayfish *P. leniusculus* and the shore crab *C. maenas*, have been studied in vitro. Results from such experiments are summarized in Table 1. They show that the different haemocyte types carry out different functions in immunity.

Moreover, cell co-operation and communication are necessary for at least some of the defence reactions and occur when a microorganism or parasite is recognized and an immune response is mounted (Söderhäll and Smith, 1986; Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1992). For example, during a fungal infection, a specific β-1,3-glucan-binding protein (βGBP) (Duvic and Söderhäll, 1990; Cerenius et al., 1994) in the plasma first recognizes and binds fungal cell walls. Then, the semigranular and the granular haemocytes respond to the βGBP complexed with glucans by degranulation (Barracco et al., 1991) and release of the proPO activating system (Söderhäll and Cerenius, 1998), including the cell-adhesive and opsonic protein, peroxinectin (Johansson and Söderhäll, 1988; Johansson et al., 1995), from storage granules. Finally, released peroxinectin can stimulate the phagocytosis by hyaline cells (Thörnqvist et al., 1994) or the encapsulation by semigranular cells (Kobayashi et al., 1990).

Granular and semigranular cells can be cytotoxic and lyse foreign eukaryotic cells. This has been shown with both tumorous and non-tumorous cell lines as well as erythrocytes as target cells (Söderhäll et al., 1985). The tumor cell targets used are especially susceptible to lysis by mammalian natural killer cells (NK cells); thus, the invertebrate cytotoxic cells may resemble mammalian NK cells. It appears that the lytic factor or factors are released during degranulation. The sugar derivative, *N*-acetylglucosamine was shown to inhibit the lytic activity in the crayfish haemolymph. Recently, an *N*-acetylglucosamine-binding factor, which is responsible for the haemolytic activity was identified and isolated from *P. leniusculus* (Wang, H. and Johansson, M.W., unpublished). This glucosamine derivative is part of many structural polymers, including those of the bacterial cell wall and fungal chitin (Prescott et al., 1996).

The haemocyte count can vary greatly in response to infection, environmental stress and endrocrine activity during moulting cycle (Smith and Ratcliffe, 1980; Persson et al.,

Table 1
Crayfish and crab haemocytes

Haemocyte type	Function in immunity
Hyaline cell	Phagocytosis[a]
Semigranular cell	Encapsulation[b]
	Phagocytosis (limited)[a]
	Storage and release of the proPO system[c]
	Cytotoxicity[d]
Granular cell	Storage and release of the proPO system[c]
	Cytotoxicity[d]

Only references to studies using isolated haemocyte populations are included.
[a]Smith and Söderhäll (1983b), Söderhäll et al. (1986), Thörnqvist et al. (1994).
[b]Persson et al. (1987a), Kobayashi et al. (1990).
[c]Johansson and Söderhäll (1985).
[d]Söderhäll et al. (1985).

1987b; Smith and Johnston, 1992). Experimental injection of a fungal cell wall preparation or of a β-1,3-glucan causes a rapid decrease in the number of free haemocytes, followed by a slow recovery (Persson et al., 1987b). Also after an injection of the crayfish parasite *Psorospermium haeckelii*, the number was significantly lower than after a control injection of saline, which, in contrast, gives a dramatic increase in the number of free haemocytes (Persson et al., 1987b; Söderhäll and Cerenius, 1992; Thörnqvist and Söderhäll, 1993). The differential haemocyte count may also vary. As an example, Sequeira et al. (1996) could distinguish the different haemocyte populations in the shrimp *P. japonicus* by flow cytometry and analyze them over the moulting cycle. In this species, the hyaline cells were seen to be the dominant population before and soon after the moult, whereas they decreased over the intermoult.

The variations in haemocyte number are, most likely, mainly regulated by release from the haematopoietic tissue, perhaps complemented by storage and release of haemocytes at other sites. Several workers have attempted to find proliferation of circulating haemocytes. Some have reported evidence for this, whereas others have not detected it. Gargioni and Barracco (1998) recently observed division of circulating haemocytes in *P. paulensis*; less than 1% of the cells had mitotic figures. Earlier, in *P. japonicus*, a small proportion (0.6%) of the circulating haemocytes were found by flow cytometry to have a double amount of DNA and were concluded to be in G2 or M phases of the cell cycle (Sequeira et al., 1996). In shrimp injected with lipopolysaccharide (LPS) or infected with the fungus *Fusarium*, this proportion was increased to about 3%; ^{3}H-thymidine uptake was also increased after LPS injections. However, in the population of cells with double amount of DNA, these authors were unable to detect dividing cells in the microscope; only cells in prophase were seen.

3. Haematopoiesis

In many crustaceans, the sheet-like haematopoietic tissue is situated on and covers the dorsal and dorsolateral sides of the stomach and is surrounded by connective tissue. Cells, believed to be haematopoietic cells, of different morphology are organized and densely packed in small lobules, and some of these morphological cell types are also found in the interlobular spaces. This situation has been found in the crab *C. maenas* (Ghiretti-Magaldi et al., 1977), the lobster *Homarus americanus* (Martin et al., 1993), and the crayfish *P. leniusculus* (Chaga et al., 1995). The arrangement is different in penaeid shrimps, e.g., *Sicyonia ingentis* (Hose et al., 1992), where haematopoiesis is believed to occur in paired epigastric haematopoietic nodules, which consist of an extensive network of vessels.

The morphology of the cells in the haematopoietic tissue in *P. leniusculus* was recently studied by electron and light microscopy (Chaga et al., 1995; see Table 2). Briefly, five morphologically different cell types were identified. At the apical part of the lobules, type 1 cells are located. These cells have the appearance of non-differentiated cells; mitosis is observed, they have no or few granules, are closely attached to other cells or to the extracellular matrix (ECM), and are difficult to liberate from the tissue. Types 2–4 cells are more distally located; they all have granules and range from

Table 2
Crayfish haematopoietic tissue cells

Cell type	Characteristics			
	Approximate proportion (%)	Mitosis	Closely attached to other cells / ECM or free	Other characteristics
Type 1	8	yes	close	semilunar shape; no or few granules
Type 2	30	yes	close or free	multiangular shape; striated granules
Type 3	55	not observed	free	elongated shape; striated granules
Type 4	2	not observed	free (?)	round shape; large refractile granules and striated granules
Type 5	5	not observed	close (?)	round shape; small dense granules

Reference: Chaga et al. (1995); extracellular matrix (ECM).

type 2 cells, which are closely attached and are the main mitotic cells in the lobules, to types 3 and 4, which have not been seen to divide, are separated from each other, easily liberated, and are also found between the lobules. The types 2–4 may represent different stages in the development of granule-containing haemocytes. Type 5 cells have granules morphologically distinct from the other types and may represent a separate lineage.

The relation between these cell types and the circulating haemocytes is not clear. Chaga et al. (1995) observed that the granules of type 4 haematopoietic tissue cells were morphologically similar to those of the circulating granular cells and that those of type 5 cells were reminiscent of the semigranular cells.

In *H. americanus* (Martin et al., 1993) and in *S. ingentis*, different types of haematopoietic tissue cells, resembling those identified in crayfish, have been described, so the general situation in these crustaceans seems to be similar. Names like granulocyte stem cell, hyaline stem cell, small granule stem cell, or even names identical to those used by the same authors for the circulating cells, such as small granule haemocyte or large granule haemocyte, have been given to these types. However, as long as the relation between the cells in the haematopoietic tissue and the circulating cells is basically unknown, such terminology indicating identity or similarity to the haemocytes may be misleading (see below).

4. Molecular characteristics of haemocytes and haematopoietic tissue

The morphologically and functionally different populations of circulating haemocytes described above have also, naturally, been found to be different at the molecular level. This has been shown clearly with separated haemocytes in several species, primarily in crayfish, using a variety of assays (Table 3). In particular, proteins, which are part of or associated with the proPO system, such as proPO and peroxinectin, are present in the semigranular and granular cells and are not detected in the hyaline cells. Other studies, which have not been performed on separated cells, but where haemocytes have been

Table 3
Molecular characteristics of crustacean haemocytes: protein expression by different cell types

Protein	Haematopoietic tissue	Haemocytes			Method
		H	SG	G	
Prophenoloxidase	−	−	+	+	In situ hybridization[a], enzyme activity[b]
Peroxinectin	?	−	+	+	Activity[c], immunogold[d], immunoblotting[e]
Cell-surface superoxide dismutase	?	?	+	+	Immunofluorescence[f]
α-Macroglobulin	?	?	+	+	Immunoprecipitation[d]
Transglutaminase	?	+	+	−	Enzyme activity[g]
Mab 40E2-2A antigen (142 kDa)	?	−	−	+	Immunofluorescence[h]
Mab 40E10-2B antigen (27 kDa)	?	+	+	−	Immunofluorescence[h]

H, hyaline cells; SG, semigranular cells; G, granular cells.
Only references to studies using isolated haemocyte populations are included.
[a]Keyser (1999).
[b]Söderhäll and Smith (1983), Smith and Söderhäll (1983b), Sequeira et al., 1996.
[c]Söderhäll et al. (1986).
[d]Liang et al. (1992).
[e]Thörnqvist et al. (1994).
[f]Johansson et al. (1999).
[g]Aono and Mori (1996).
[h]Rodríguez et al. (1995).

stained for phenoloxidase activity, have come to the same conclusion (e.g., Hose et al., 1987; Lanz et al., 1993). Monoclonal antibodies against *P. japonicus* haemolymph have been generated (Rodríguez et al., 1995); some of these can distinguish between separated haemocyte populations.

In only one case so far has the haemopoietic tissue been studied with molecular methods (Keyser, 1999). Using in situ hybridization, the haemopoietic tissue was found to be negative for proPO, whereas the majority of the haemocytes were clearly positive. This shows that although some of the cells in the haematopoietic tissue morphologically may resemble granular or semigranular cells, their protein expression is different. Since most of circulating haemocytes express proPO, the onset of proPO production must be immediately before or after the release from the haematopoietic tissue. It is also possible that the production of proPO in the tissue is too low to be detected. However, it suggests that the cells in the haematopoietic tissue should not be labeled with the same name as the circulating haemocytes.

More molecular studies using, for instance, in situ hybridization or monoclonal antibodies, in particular on the haematopoietic tissue cells, are warranted in order to clarify the connection between, and the development and maturation of the different populations of haematopoietic tissue cells and circulating haemocytes.

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