

The haemocytic origin of lymphoid organ spheroid cells in the penaeid prawn *Penaeus monodon*

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ABSTRACT: Studies on lymphoid organ spheroid (LOS) cells of *Penaeus monodon* were undertaken. Phenoloxidase and peroxidase assays showed that LOS cells have characteristics similar to semi-granular and, in particular, large granular haemocytes. The mean percentage of LOS cells positive for phenoloxidase and peroxidase was 85 ± 23 and $82 \pm 23\%$, respectively. There was no significant difference between the sites of phenoloxidase and peroxidase activity in LOS cells ($t = 1.617$, $df = 29$, $p > 0.05$). The relative sectional area occupied by LOS cells relative to that of the stromal matrix cells from both laboratory-held and farmed prawns was not correlated to increasing weight or total length of the prawns ($p > 0.05$). An apoptosis detection assay showed that LOS cells were often apoptotic whilst stromal matrix cells were not. There was a significant difference ($t = -5.533$, $df = 58$, $p < 0.05$) in the mean percentage of apoptotic spheroid cells between laboratory-held prawns ($52 \pm 24\%$) and farmed prawns with midcrop mortality syndrome (MCMS) ($80 \pm 12\%$). In conclusion, LOS cells have the characteristics of exocytosed, granular haemocytes that have phagocytosed foreign material, particularly viruses, and probably constitute a major mechanism for penaeid antiviral defense.

KEY WORDS: *Penaeus monodon* · Haemocyte · Lymphoid organ · Spheroid cells · Prophenoloxidase · Peroxidase · Apoptosis · Midcrop mortality syndrome

INTRODUCTION

Oka (1969) first described the lymphoid organ (LO) in the penaeid prawn *Penaeus orientalis*, and later Martin et al. (1987) incorrectly described the LO structure as haematopoietic nodules in the ridgeback prawn *Sicyonia ingentis*. Bell & Lightner (1988) described the normal structure of LO of penaeid shrimp. It was generally believed that the LO in penaeids played an important role in the immune response (Kondo et al. 1994, Martin et al. 1996). Studies by Martin et al. (1996) on the role of lymphoid tissue (incorrectly termed haematopoietic tissue) of ridgeback prawn *S. ingentis* revealed that lymphoid nodules were the most specific and effective organ for bacterial clearing. Special tissue changes in penaeid LO called spheroids have been associated with viral infection, i.e. 'Nakamura organ' hypertrophy and metastasis (Lightner et al. 1987), lymphoidal parvo-like virus (Owens et al.

1991, 1992), LO vacuolization virus (Bonami et al. 1992), rhabdovirus of penaeid shrimp (Nadala et al. 1992), yellow head virus (Boonyaratpalin et al. 1993), LO virus (Spann et al. 1995), spawner-isolated mortality virus (Fraser & Owens 1996) and Taura virus (Hasson et al. 1995). Besides normal LO matrix cells, Lightner et al. (1987) found in the 'Nakamura (lymphoid) organ' of *Penaeus monodon* and *Penaeus penicillatus* that there were extremely active spheroid clumps of what were described as hyperplastic tubules that lacked a central vessel. Lightner (1996) also proposed that *Penaeus vannamei* infected with LO vacuolization virus showed LO spheroids comprising disorganised LO tubules that lacked a central vessel and consisted of cells that showed karyomegaly, large prominent cytoplasmic vacuoles and other cytoplasmic inclusions. Moreover, Owens (unpubl. data) has classified changes in lymphoid organ spheroid (LOS) cells into 3 phases. First is a tumorous-like phase that does not have any encapsulating fibrocytes around the spheroids (as described in spawner-isolated mortality virus infection by Fraser & Owens (1996)). Second is a fully

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encapsulated spheroid phase as described in lymphoid parvo-like virus infection (Owens et al. 1991) and LO virus infection (Spann et al. 1995). Third is vacuolative degeneration of spheroids which was reported first by Owens & Hall-Mendelin in 1989 (Owens & Hall-Mendelin 1990) but was also described in LO vacuolization virus infections (Bonami et al. 1992). Lightner et al. (1987) also described ectopic metastatic spheroids in the cuticular hypodermis of *P. vannamei*. Despite the detection of several disease agents in the LO of prawns, its role, particularly with respect to spheroid cells, still remains unclear.

Haemocytes are considered the primary line of internal defence against infection in Crustacea, and they have therefore been studied in some detail. The crustacean cellular defence system consists of sequential coagulation, phagocytosis, encapsulation, and nodule formation (Fontaine & Lightner 1974, Bauchau 1981, Soderhall & Cerenius 1992). Once stimulated by foreign particles (Song & Hsieh 1994), haemocytes can produce highly reactive oxygen radicals such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxide ions (OH^-). According to Deimann (1984), endogenous peroxidase can be visually located in tissues by its catalytic H_2O_2 oxidation of DAB (3,3-diaminobenzidine tetrahydrochloride). The prophenoloxidase system, besides producing highly reactive oxygen radicals, also activates certain types of haemocytes. Through an enzyme cascade, prophenoloxidase is converted into phenoloxidase (PO) in the terminal phase. The latter participates in cellular defence by opsonising cells for encapsulation (Johansson & Soderhall 1985, 1989, Aspan & Soderhall 1991, Kondo et al. 1992), and by participating in melanization reactions (Soderhall & Cerenius 1992, Adams 1994), nodule formation (Smith et al. 1984, Adams 1994), and phagocytosis (Smith & Soderhall 1983, Soderhall et al. 1986, Kondo et al. 1992, Adams 1994). PO activity can be detected by observing the formation of dopachrome from L-DOPA (dihydroxyphenylalanine).

This paper describes the role of haemocytes in the production of LOS cells in penaeid prawns (the term 'spheroid cells' will be used throughout the rest of the paper).

MATERIAL AND METHODS

Preliminary studies. Preliminary studies were conducted to evaluate and modify techniques that could be applied easily to the LOs of prawns. Included were the preparations of smears and sections, such as impression smears (Vickers et al. 1993), frozen sections (Lillie & Fulmer 1976), paraffin-embedded tissue sections (Bell & Lightner 1988) and different staining pro-

cedures for detecting oxygen free radicals using nitroblue tetrazolium (Song & Hsieh 1994), and 3,3-diaminobenzidine tetrahydrochloride (Fahimi 1979, Sequeira et al. 1995). The following procedures describe the methods which gave the best results.

Prawns. The prawns used in all trials were *Penaeus monodon* from commercial farms in northern Queensland, Australia. For the 'area analysis' study, 20 prawns which had been kept for over 1 mo in the laboratory at James Cook University and had a final weight of 8.1 ± 3.3 g and a total length of 10.9 ± 1.3 cm were compared to 20 prawns from a different commercial farm with a final weight of 8.3 ± 5.0 g and a total length of 10.0 ± 1.8 cm.

Thirty prawns which had been kept for over 1 mo in the laboratory and had a final weight of 7.5 ± 3.1 g and a total length of 10.3 ± 1.1 cm were used for assessment of PO activity, peroxidase activity, *in situ* hybridization assay and apoptosis detection assay. From a commercial farm, another 30 prawns which had been diagnosed by the Oonoonba Veterinary Laboratory as suffering from midcrop mortality syndrome (MCMS) and had a final weight of 7.9 ± 4.7 g and a total length of 8.5 ± 2.1 cm were also tested for apoptotic cells. These prawns were gene-probed by *in situ* hybridisation to Spawner-isolated Mortality Virus (SMV) (Owens et al. 1998) and found to be positive to this virus. SMV is one of a suite of viruses found in prawns affected by MCMS.

Paraffin-embedded tissues. The prawns were preserved in Davidson's fixative (Bell & Lightner 1988) by injecting 0.5 ml of the fixative directly into the hepatopancreas and adjacent areas of the cephalothorax of each prawn. After 24 h in Davidson's fixative, the cephalothorax was cut in half longitudinally, placed in a histocassette, transferred to 70% ethanol, and then processed for routine histological examination using standard paraffin-embedded procedures as described by Bell & Lightner (1988).

Peroxidase activity assay. The peroxidase assay was adapted from Sequeira et al. (1995), who observed the peroxidase activity in haemocytes of *Penaeus japonicus*. Five μ m tissue sections were placed on a glass slide, incubated at 60°C for 1 h, dewaxed in xylene and absolute ethanol, washed by immersion in 0.1 M Tris-HCl buffer (pH 7.4), and then incubated in the dark in 0.1 M Tris-HCl buffer containing 2 mg ml⁻¹ DAB and 0.003% H_2O_2 for 3 h at 30°C. The sections were examined under a light microscope, and dark brown to black staining of granules was interpreted as presence of peroxidase activity.

Prophenoloxidase activating system assay. Prawn tissues were cut at 5 μ m, placed on a silanized glass slide, incubated at 60°C for 1 h, dewaxed in xylene and absolute ethanol, and then tested for PO activity using

the method of Hose et al. (1987), who observed what he called the pro-PO system in haemocytes of the ridgeback prawn *Sicyonia ingentis*. Tissues were washed by immersion in 0.1 M phosphate buffered saline (PBS) (pH 7.4) for 1 min, post-fixed in 2.5% glutaraldehyde in 0.1 M PBS for 1 h at 4°C, rinsed 3 times for 5 min each in 0.1 M PBS, incubated in 0.1% L-DOPA in 0.1 M PBS for 16 h in the dark at room temperature, and then examined by light microscopy for dark brown staining of the granules interpreted as PO activity.

Area analysis. After embedding in paraffin, tissues were cut at 5 µm and stained with H&E. The area of spheroid cells and stromal matrix cells was observed by light microscopy and analyzed using Jandel Scientific Software: Sigma Scan and Sigma Scan Pro (image measurement software and automated image analysis software).

In situ hybridization. The *in situ* hybridization probe used for this assay was derived from the prawn 18S rRNA gene and prepared by Dr Catriona McElnea (Department of Microbiology and Immunology, James Cook University) based on published sequences by Kim & Abele (1990). The protocol was adapted from HPV-IS kit for hepatopancreatic parvovirus from DiagXotics, Inc., Wilton, CT, USA, with the following modifications. Tissue sections were permeabilised with 100 µg ml⁻¹ Proteinase K for 10 min at 56°C and then slides were washed with sterile water following stabilisation with cold 0.4% paraformaldehyde and allowed to air dry. The 18sRNA probe was then applied to the slide at 1 ng µl⁻¹ and both template and probe DNA denatured simultaneously at 95°C for 6 min before quenching on ice and hybridising overnight at 42°C. The sections were mounted with DPX (dibutylphthalate-polystyrene-xylene) and examined by light microscopy.

Apoptosis detection. Detection of apoptotic cells by TUNEL (TdT-mediated dUTP nick-end labelling) assay was conducted using a Promega kit no. G. 325 'Apoptosis Detection System, Fluorescein', as directed in the kit manual. The samples were examined by fluorescence microscopy at 520 ± 20 nm.

Statistical analysis. From each prawn, 300 randomly selected cells were counted for statistical analysis of PO, peroxidase activity sites in the cells, and also for apoptotic cells. Paired sample *t*-test was used for comparing the mean percentage of spheroid cells that were PO positive and peroxidase positive, whilst independent-sample *t*-test was used for testing the mean percentage of apoptotic spheroid cells of laboratory-held prawns compared to prawns with MCMS. SPSS™ 8.0 program was used for conducting the *t*-test and alpha was 0.05. Statistical correlations were also determined using the SPSS program.

RESULTS

PO and peroxidase

The results of PO and peroxidase tests on the LO of *Penaeus monodon* (Figs. 1 & 2 respectively) showed PO and peroxidase activity was localized in spheroid cells and seen as dark brown to black granule staining. By contrast, the stromal matrix cells remained unstained or showed pale brown staining. From 300 counts of LO cells, the mean percentage of spheroid cells positive for PO and peroxidase was 83.5 ± 23.3% and 81.6 ± 22.9%, respectively. There was no significant difference in the number of sites for PO and peroxidase activity in the spheroid cells ($t = 0.117$, $df = 29$, $p > 0.05$). There was no correlation between the percentage of PO and peroxidase positive spheroid cells and weight of the prawn ($r = -0.075$ for PO and $r = -0.006$ for peroxidase).

In situ hybridization

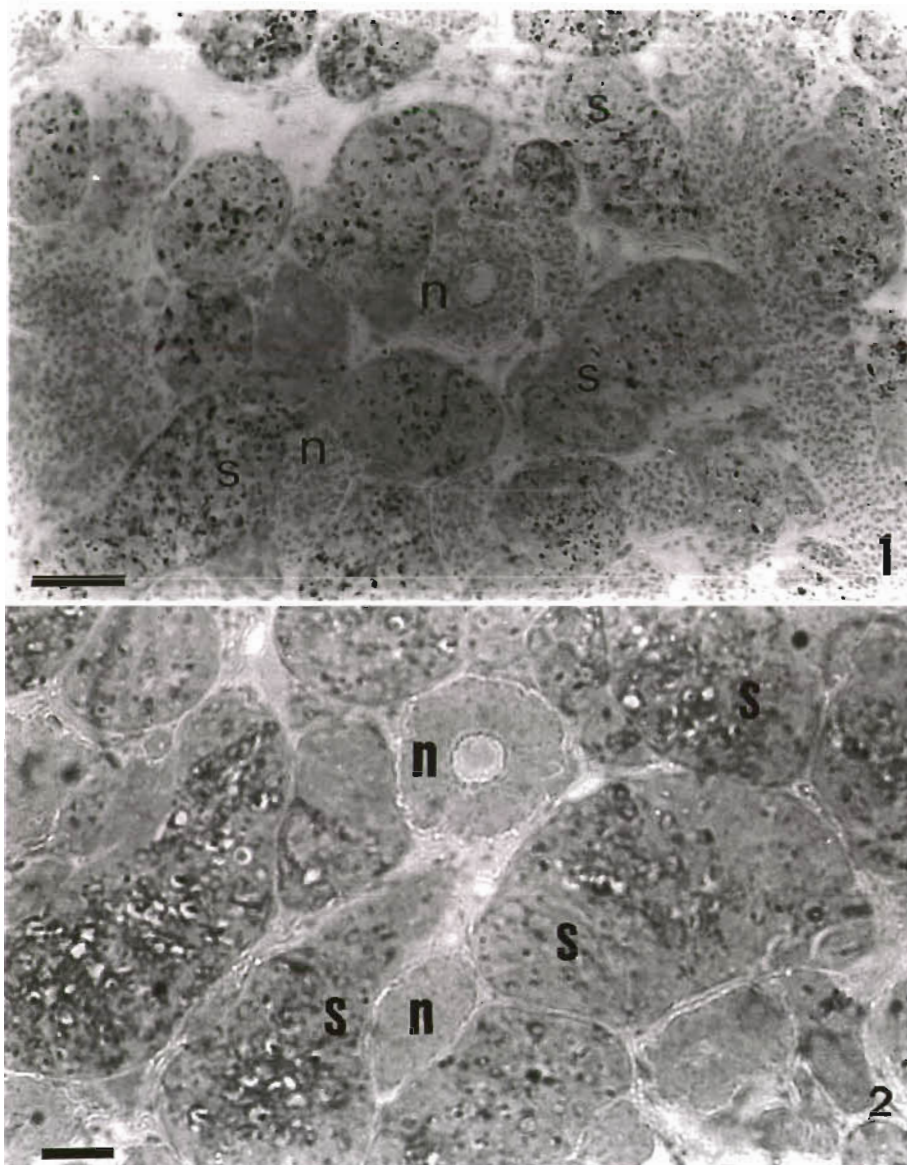
In situ tests with the rRNA probe showed that spheroid cells were explicitly differentiated by strong, blue hybridization signals from the pink staining stromal matrix cells (Fig 3). This indicated that spheroid cells had high 18S rRNA translating activity, suggestive of a higher metabolic rate than the stromal matrix cells. The lumens of LO tubules often contained haemocytes and tubule endothelial cells sometimes stained blue.

Apoptosis

Both batches of prawns, those kept in the laboratory and those diagnosed with MCMS, sometimes showed apoptotic spheroid cells. The green colour of apoptotic spheroid cells due to fluorescein-12-dUTP staining was stronger in prawns with MCMS (Figs. 4 & 5) and the mean percentage of apoptotic cells for them was significantly higher ($t = -5.533$, $df = 58$, $p < 0.05$) at 79.9 ± 12.3% than for laboratory-held prawns (Fig. 6) at 52.5 ± 24.2%.

Area analysis

Quantitative analysis of spheroid cells showed that there was no significant ($p > 0.05$) correlation between area and prawn length or weight. Similarly, there was no significant ($p > 0.05$) correlation between the ratio of spheroid cell area to total LO area and prawn length or weight. This lack of correlation was demonstrated for both laboratory-held and farmed prawns. However,



Figs. 1 & 2. Light photomicrographs of lymphoid organ of *Penaeus monodon* stained with DAB (3,3-diaminobenzidine tetrahydrochloride) and with L-DOPA (dihydroxyphenylalanine) from one prawn on the same lymphoid organ site. **Fig. 1.** Spheroids (s), seen here as gray with darker gray and black spots, gave an overall brown colour and contained dark brown to black spots, indicative of a positive peroxidase reaction with DAB. Normal tubules (n), seen here as lighter gray, remained unstained or stained very-lightly yellow-brown. Scale bar = 100 μ m. **Fig. 2.** Spheroids (s), seen here as dark gray with darker gray and black spots, gave an overall dark brown colour and contained darker brown to black spots, both indicative of a positive phenoloxidase (PO) reaction with L-DOPA. Normal tubules (n), seen here as light grey, were unstained or very pale yellow-brown. Scale bar = 60 μ m

the laboratory-held prawns had a significantly higher area of spheroid cells ($t = 3.52$, $df = 38$, $p < 0.05$) and proportion of spheroid cells ($t = 4.25$, $df = 36$, $p < 0.05$) than did the farmed prawns.

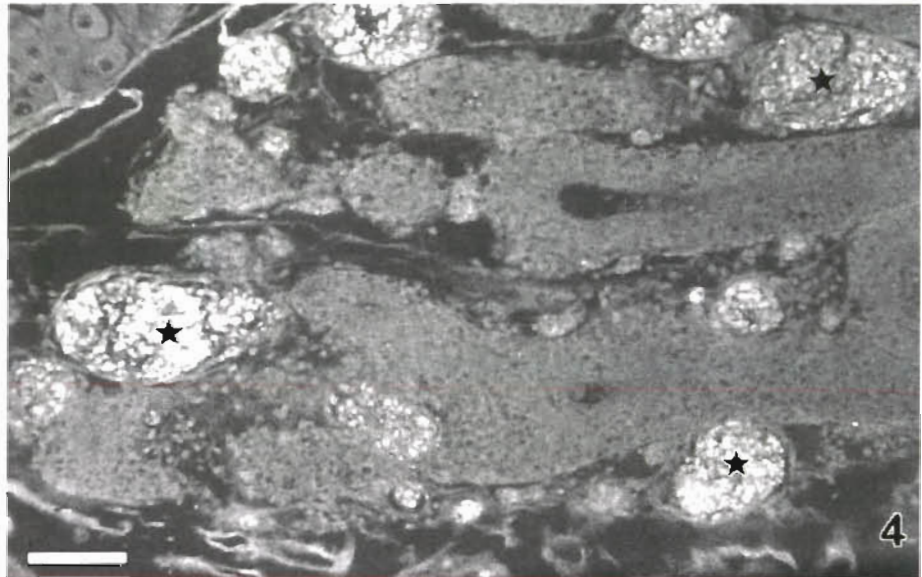
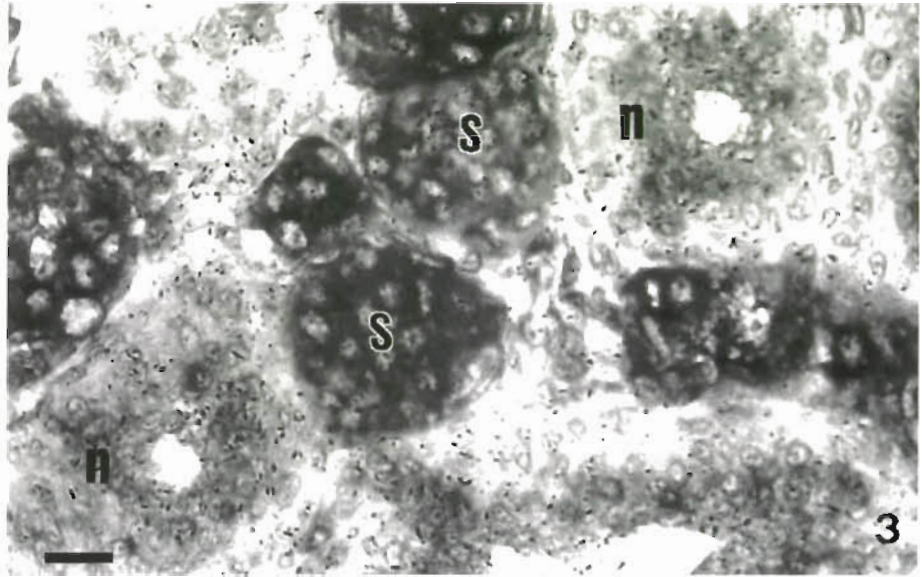
DISCUSSION

Researchers characterizing crustacean haemocytes have used several different criteria and terminologies. Morphologically, they can be categorised by the presence or absence of granules and this was used in early studies of penaeid haemocytes, as in *Metapenaeus mastersii* (Dall 1964), *Sicyonia ingentis*, *Penaeus californiensis* (Martin & Graves 1985), and *Penaeus japonicus* (Tsing et al. 1989). Martin & Graves (1985)

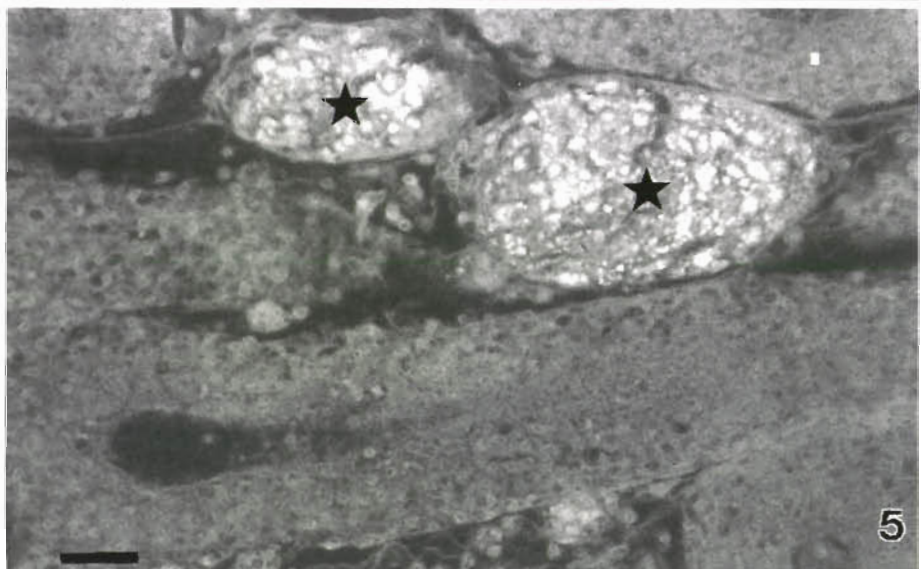
classified them as agranular, semi-granular or large granular haemocytes. Similarly, Tsing et al. (1989) classified them as undifferentiated haemocytes (UH), small granular haemocytes (SGH) or large granular haemocytes (LGH). Neither the term 'agranular' nor the term 'undifferentiated' is correct. We will use Tsing's classification here but use the word hyalinocyte (HC) for those cells that have no obvious granules under light microscopy, as this classification is the least presumptive.

Cytochemical studies on *Sicyonia ingentis* haemocytes by Hose et al. (1987) revealed that prophenoloxidase was localised in SGH and in LGH. In *Penaeus japonicus*, Sequeira et al. (1995) found that PO activity in both SGH and LGH but that peroxidase activity was confined to LGH. In addition, Sung et al. (1998) re-

Fig. 3. Photomicrograph of a section of lymphoid organ of *Penaeus monodon* hybridized with a gene probe for the prawn 18S rRNA gene. Spheroid cells (s), seen here as dark gray to black, stained dark blue, indicating large quantities of rRNA. The staining contrasted sharply with the stromal matrix cells of the normal tubules (n), seen here as a lighter gray, but staining lightly pink with the counter stain, indicating a low quantity of rRNA. Stained with DIG and counter stained with neutral red. Scale bar = 20 μ m



Figs. 4 & 5. Photomicrograph of the lymphoid organ of *Penaeus monodon* diagnosed with mid-crop mortality syndrome (MCMS) (see 'Material and methods'). Seen here as bright white against a dark background, nuclei stained with fluorescein-12-dUTP gave a strong green fluorescence and were present in high numbers in spheroids (★), indicating a high number of cells undergoing DNA fragmentation, characteristic of apoptosis. By contrast, nuclei from normal tubules, seen here as darker gray, stained red/orange with propidium iodide, indicating no fragmented DNA. Scale bars: Fig. 4 = 100 μ m, Fig. 5 = 40 μ m



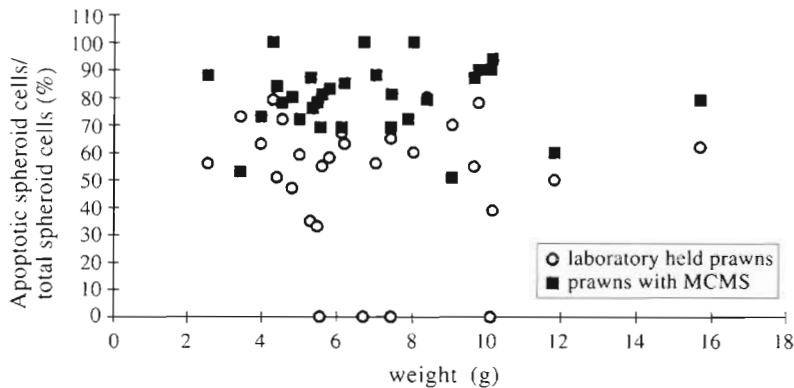


Fig. 6. Relationship between ratio of apoptotic spheroid cells to total spheroid cells in normal, laboratory-held *Penaeus monodon* and farmed *P. monodon* with MCMS. Prawns with MCMS had significantly higher ratios than normal prawns ($p < 0.05$). However, there was no correlation between these ratios and prawn weight ($p > 0.05$) for either group

ported that the prophenoloxidase system existed in the granulocytes of tiger shrimp *Penaeus monodon* and the giant freshwater prawn *Macrobrachium rosenbergii*. However, the staining protocol they used could not determine whether the prophenoloxidase system was present in SGH. On the other hand in crayfish, Thornqvist & Soderhall (1997) have shown that prophenoloxidase and inactive peroxinectin are present in both SGH and LGH granules and that they are specifically activated upon exocytosis. Peroxinectin exhibits both opsinin and peroxidase activities (Johansson et al. 1995). Obviously, the ability to measure these activities depends upon whether the zymogens have been activated or not. In summary, these papers suggest that in penaeids HC have no granules and no PO or peroxidase activity. Small granular haemocytes have granules and prophenoloxidase but no peroxidase. Large granule haemocytes have granules, prophenoloxidase and peroxidase. However in crayfish, both SGH and LGH have both PO and peroxidase.

Cytochemically, the present study showed that spheroid cells of *Penaeus monodon* had similar characteristics to SGH and LGH due to their staining for PO and peroxidase activity. Morphologically, by light microscopy, spheroid cells were not granulated. Thus, if they originated from granular haemocytes, then exocytosis of granules would have had to occur in another site or during spheroid formation and they would then consist of spent haemocytes. Given our results and the background information, we feel that the most parsimonious interpretation is that LOS cells are exocytosed (i.e., spent) granular haemocytes.

Research by Martin & Hose (1992) on LO of *Sicyonia ingentis* showed that haemocytes must pass the thin intimal layer of endothelial cells to enter the circulation and that all of the 3 types of haemocytes are present in the tubule wall and embedded in stromal matrix cells.

Following this, Martin et al. (1996) showed that injection of radiolabelled bacteria into the haemolymph of *S. ingentis* resulted in its localisation in gills, heart, abdominal musculature and what they called haematopoietic nodules but what are obviously LO by our interpretation of the published figures of Martin & Hose (1992). They found the latter the most specific and effective organ for bacterial clearance. There is no evidence of high mitotic activity in either the matrix or the spheroid cells, suggesting that the LO is not a haematopoietic tissue.

Thus, our conclusions differ from those of Martin & Hose (1992). The fact that stromal matrix cells showed limited staining for peroxidase and PO activity,

whilst spheroid cells were extremely positive, could be interpreted in several ways, but we favour the following hypothesis: We propose that LOS cells have a haemocytic origin and that matrix cells do not. We suggest that spheroid cells begin as haemocytes that migrate from the LO tubule lumen, through the stromal matrix as phagocytic cells, contributing to the phagocytic nature of the stromal matrix. We further hypothesise that this is part of the normal haemocyte migratory route from the dorsal aorta and heart, through the stromal matrix area into the haemal sinuses behind the stromal matrix and thence to the main haemocoel. This is consistent with the haemolymph flow but opposite to the direction of cell movement proposed by Martin & Hose (1992). Our supposition is supported by the work of Kondo et al. (1994), who showed that injected substances localised immediately in the lymphoid arteriolar tubules and only months later in sinusoidal spheroids.

Spheroid cells have not been associated with bacterial infections in any published work that we are aware of. Conversely, spheroid cells have been found associated with many, if not all, systemic viral infections, i.e., 'Nakamura organ' hypertrophy and metastasis (Lightner et al. 1987), lymphoid parvo-like virus (Owens et al. 1991, 1992), LO vacuolization virus (Bonami et al. 1992), rhabdovirus of penaeid shrimp (Nadala et al. 1992), yellow head virus (Boonyaratpalin et al. 1993), LO virus (Spann et al. 1995, 1997), spawner-isolated mortality virus (Fraser & Owens 1996) and Taura virus (Hasson et al. 1995). Therefore, we suggest that spheroid cells represent a major mechanism for sequestering viruses in penaeid prawns.

Apoptosis plays an important role in the defence system by removing unwanted and potentially dangerous cells such as self-reactive lymphocytes, virus-infected cells and tumour cells (Wyllie et al. 1980, Raff 1992,

Martin et al. 1994, Steller 1995). According to our TUNEL assays, the numbers of apoptotic spheroid cells were higher and the TUNEL assay signal was stronger in LO of prawns with MCMS. However, this requires further study, since the apoptosis detection kit labels the 3'-OH end of DNA in general, and this may include viral DNA in addition to fragmented DNA of apoptotic cells. Thus, there was a slight possibility that viral DNA inside the spheroid cells was TUNEL labeled, resulting in an erroneously high percentage of apparently apoptotic cells in prawns with MCMS. Indeed many viruses have been found to replicate in the spheroids of prawns (see above). A major implication of this work is that prawns must be capable of viral recognition for phagocytosis and sequestering to occur.

It is well known that the stages of the moulting cycle in Crustacea influence their physiology, behaviour, reproduction, and immunity. The moulting cycle of penaeid prawns can be divided into 4 main stages (Schafer 1968, Read 1977, Huner & Colvin 1979, Longmuir 1983, Robertson et al. 1987). These stages are: early postmoult (Stage A), late postmoult (Stage B), intermoult (Stage C), and premoult (Stage D₀, D₁, D₂, D₃, including ecdysis as Stage E). There was a correlation between immunological state and moult stage in penaeid prawns including *Penaeus japonicus* (Tsing et al. 1987, Sequeira et al. 1995) and *Penaeus stylirostris* (LeMoullac et al. 1997). Sequeira et al. (1995) found that the percentage of SGH and LGH increased during intermoult (Stage C) in both female and male prawns and during premoult (Stage D₀) in female prawns. The percentage of hyaline haemocytes declined in both female and male prawns at these times. PO activity measured in haemocytes was also significantly higher in intermoult than in premoult (LeMoullac et al. 1997). Further study on moult cycle of *Penaeus indicus* by Vijayan et al. (1997) showed that moult cycle duration was greater with increasing animal size. If spheroid cells accumulated in haemal sinuses of LO over the life of a prawn, the area of spheroid cells would be expected to increase with increasing prawn size. However, the area of spheroid cells was not correlated to any measure of size of the prawn. It is hypothesised that the spheroid cells are disposed of, possibly with moulting, and this hypothesis is now being investigated.

In conclusion, we propose that lymphoid spheroid cells have the characteristics of exocytosed, granular haemocytes that have phagocytosed foreign material, particularly viruses, and that they are probably the major mechanism for penaeid antiviral defense.

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