Cellular response to injury in spiny lobsters

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ABSTRACT

This paper presents a review of the cellular defense mechanisms of spiny lobsters. These mechanisms can be divided, for convenience, into three broad groupings: maintenance of exoskeleton integrity; foreign agent recognition, inactivation and elimination from the internal organs; and repair of damage by toxins. Cellular defense mechanisms are dependent on circulating haemocytes and phagocytes, fixed phagocytes and fibrocytes. The process or processes by which these cell types are generated and mature in the animal have not yet been adequately described for spiny lobsters. In addition, attention has only recently focused on the way in which cellular defence responses are influenced by environmental stress and by the nutritional and moult status of the lobster. These are areas of critical importance to animal husbandry and production in aquaculture. While rapid advances are being made in the understanding of humoral defense mechanisms of crustaceans there are still large gaps in our understanding of the cellular components of the system in spiny lobsters.

Key Words: review, encapsulation, haemocytes, lobster, Panuliridae, phagocytes.

I. INTRODUCTION

Understanding of the internal defense mechanisms of crustaceans began in the 1880's through the pioneering work of Metchnikoff on phagocytosis and the inflammatory process. Likewise, Cantacuzène, in a series of papers between 1912 and 1934 started investigation of the humoral defense responses. Work then languished until the 1960's (Sinderman 1971) when interest was renewed as research began on crustacean diseases of major economic significance, particularly Gaffkaemia in lobsters and fungal infections of freshwater crayfish.

Cellular defense mechanisms in spiny lobsters (Panuliridae) have seldom been studied directly, but inferences can be drawn from apparently similar mechanisms in other decapods (Tsing *et al.* 1989). These cellular defense mechanisms can be divided, for convenience, into three broad groupings: maintenance of exoskeleton integrity; foreign agent recognition, inactivation and elimination from the internal organs; and repair of damage by toxins. These systems are not mutually exclusive but share five basic processes: phagocytosis; haemocytosis; degranulation; coagulation (clotting); and encapsulation. Cellular defense mechanisms are particularly dependent on circulating haemocytes, fixed phagocytes and fibrocytes.

Maintenance of exoskeletal integrity

The chitinous exoskeleton of spiny lobsters is an effective barrier that prevents the entry of infectious agents as well as providing muscle anchorage and protecting underlying soft tissue. The first barrier presented by the exoskeleton against invasion is the very thin proteolipid epicuticular membrane or 'surface waxy layer' (Unestam 1973, Malloy, 1978, Fisher 1988). Beneath this layer is the calcified exocuticle. This is very difficult to penetrate, even for disease agents secreting extracellular chitinases. By contrast, the soft non-calcified endocuticle is easily penetrated by such agents (Unestam 1973). Maintenance of the epicuticle is dependent on diet (Fisher *et al.* 1976) and it is probable that penetration is also related to the nutritional status of the animal. Shell diseases are a characteristic of crustaceans held in captivity (Stewart 1993).

Rapid sealing of wounds to the exoskeleton is required to prevent loss of haemolymph and minimize opportunistic invasion. Reactions leading to wound repair in spiny lobsters consist of rapid haemocyte accumulation and aggregation at the wound site followed by intravascular clotting. Clotting is initiated by contact of hyalinocytes with seawater (Hose & Martin 1989). The clot results from direct conversion of a soluble fibrinogen (coagulogen) into crosslinked fibrin through the action of a coagulin released by haemocyte rupture (Fuller & Doolittle 1971a,b; Durliat & Vranckx 1981; Ghidalia *et al*. 1981; Hose *et al*. 1990; Aono & Mori 1996). This is followed by melanisation of the wound area to form a dense black membrane beneath which the new epidermis forms. Melanin is produced by the action of the enzyme polyphenoloxidase on melanin precursors (Unestam & Nylund 1972; Bauchau 1981) and has antimicrobial properties (Nyhlen & Unestam 1980; Söderhäll & Ajaxon 1982). The epidermis involutes into the wound utilizing the haemocyte network as basal support. New cuticle is formed by this epidermal layer and lies beneath the melanin membrane (Fontaine 1975). In association with the haemocyte response a dense network of collagen-like fibres forms. This fibrous tissue is not resorbed but remains as a scar (Fontaine & Lightner 1975).

Foreign agent recognition, inactivation and elimination

Foreign agent recognition, inactivation and elimination is effected through both cellular and humoral host defence responses. Immunorecognition is thought to be mediated through the prophenoloxidase system, a cascade of serine proteases and prophenoloxidase present in the haemocytes which is activated by the presence of non-self molecules and initiates melanization (Söderhäll & Smith 1986; Söderhäll *et al.* 1996). Subsequent host defence responses comprise cellular mechanisms together with humoral responses involving the actions of circulating antibacterial factors, lectins and other immunologically active molecules. Invertebrates do not exhibit acquired immunity (Roch 1999) although proteins, with domains belonging to the immunoglobulin superfamily, have been demonstrated (Lanz Mendoza & Faye 1996). Humoral responses will not be discussed further.

The relative importance of cellular and humoral host defence mechanisms in spiny lobsters has yet to be determined. It would seem, however, that circulating haemocytes play a central role in both through their involvement in immunorecognition and in the processes of inactivation and elimination.

Spiny lobster haemocytes

Spiny lobsters, as with most crustaceans, have at least three recognised blood cell types based on morphology and staining characteristics (Hearing & Vernick 1967; Hose *et al*. 1990; Jussila *et al*. 1998): granulocyte (large granule haemocyte; eosinophil); hyalinocyte; and semi-granulocyte (small granule haemocyte). Semi-granulocytes and granulocytes adhere readily to glass and plastic and emit long filopodia, assuming a stellate shape as described by Newman & Feng (1982) for haemocytes of *Cancer irroradiatus*, by Goldberg *et al*. (1984, 1986) for *Homarus*, and by Barracco & Amirante (1992) for *Squilla mantis*. Filopodia are apparently associated with surface adherence and the density and type of cytoplasmic granules in the haemocyte (Goldberg *et al*. 1986).

It has been generally accepted, but not proved, that the three types represent different developmental stages of one cell line, with the granulocyte being the terminal stage (Bodammer 1978; Mix & Sparks 1980; Jussila *et al*. 1998). It should be noted, however, that Cornick & Stewart (1978) described four cell types in *Homarus americanus*, based on cell histochemistry and morphology. They described two hyalinocye types and two granulocytes, one eosinophilic and one chromophobic. Williams & Lutz (1975) presented evidence for five types in *Carcinus maenas*, based on cell histochemistry. They divided granulocytes into two classes based on whether the granules stained for glycogen or not. Barracco & Amirante (1992) found two subgroups of semi-granulocytes in *Squilla mantis* (Stomatopoda) which were ultrastructurally distinct. Johnston *et al.* (1973) reported two haemocyte cell lines in *Carcinus maenas*, those they called *alpha* cells have a strongly basophilic nucleus and form a single developmental series from small ribosome-rich non-granular cells to large carbohydrate laden haemocytes. They describe *beta* cells as large with a moderately acidophilic nucleus when stained with Wright's stain and there is a uniform and intense acidic granulation of the cytoplasm, and they are rarely seen intact in blood samples. Hose & Martin (1989) found that hyalinocytes initiate coagulation and they lyse in the presence of bacterial toxins and seawater, while granulocytes and semigranulocytes are involved with phagocytosis and encapsulation. If there is only one cell line, and that has yet to be established, then the changes in morphology and histochemistry as the cell matures need to be much more rigorously defined.

The location and function of the haematopoetic tissue is not well understood. In *Nephrops norvegicus* it is believed to be a thin sheet of tissue, on the dorsal and lateral surfaces of the cardiac stomach, and probably on the floor of the cephalic cavity, that shows a marked seasonal cycle of activity (Field & Appleton 1995). The location in prawns and shrimps is better known (Bell & Lightner 1988) and haematopoetic tissue has been found to occur in the same locations in spiny lobsters (pers. obs.). However, it would seem that the ultrastructure, cytochemistry and activity of haematopoietic tissue in spiny lobsters is a neglected field.

Cellular defense mechanisms - foreign agents

Inflammation has been studied in considerable detail in penaeid shrimp, and the process appears to be identical in spiny lobsters (Martin *et al*. 2000). Injection of shrimp with carmine (a neutral contaminant) is followed by accumulation of carmine in the dorsal abdominal artery, ventral abdominal vein, heart and gills. By 30 h post-injection carmine is only visible in the gills, heart and injection site (Fontaine & Lightner 1974). Histologically

the carmine forms tightly packed extracellular masses, at the injection site, which are infiltrated and phagositised by haemocytes. Circulating carmine particles are then trapped by fixed phagocytes lining the blood vessels and in sinusoids of the gill filaments. These particles finally accumulate in the distal gill filaments and heart. Brown melanised nodules consisting of necrotic haemocytes containing phagositised carmine develop in the periopods and as cysts in the connective tissues of the gill cover by a process of filtration rather than through the action of fixed phagocytes and are subsequently shed at moulting (Martin *et al*. 2000). Carmine containing haemocytes also migrate through the midgut epithelium and into the lumen of the antennal gland. Smith & Ratcliff (1980a,b) studied clearance of foreign agents from gills of the crab *Carcinus maenas*. They found that there were two mechanisms in operation: aggregation of haemocytes into 12-25 μm diameter clumps of 5 to 50 haemocytes containing trapped bacteria; and the formation of elongate, diffuse networks of phagocytic haemocytes in the gill blood sinuses.

Aggregation of semi-granulocytes and granulocytes is accomplished by a combination of binding by pseudopodia and humoral factors. This occurs in response to foreign agents such as *Vibrio* sp. (Johnson 1976; Newman & Feng 1982) and is the precursor to encapsulation for foreign agents too large to phagocytise. Aggregation is often accompanied by extensive pre-mortem clotting of plasma and, in severe cases, the aggregation and plasma clotting can obstruct haemolymph leading to massive focal necrosis (Johnson 1976).

Phagocytosis is a defense employed when the foreign agent is smaller than the haemocyte. For larger particles, a multicellular defense is required to encapsulate the agent. Encapsulation reactions by haemocyte preparations obtained from *Panulirus interruptus* were studied by Hose *et al*. (1990) who showed that the reaction involved the semi-granular haemocytes and fibrocytes. Haemocytes (granulocytes and semigranulocytes) cluster around the foreign body, forming encapsulations many cell layers thick. The outer cells retain a more normal shape while inner cells become flattened. Diffuse melanisation occurs in the compact core and in the intracellular matrix forming a thick brown leathery capsule. Such capsules are not resorbed. Haemocytes also cluster around clots which are presumably resolved in time.

Degranulation is also a neglected area of study in spiny lobsters. Observation of histological material shows that mature granulocytes appear to aggregate near foreign agents and degranulate in the same way as molluscan haemocytes. This process was studied in quahog *(Mercenaria mercenaria*) by Mohandas *et al*. (1985) who showed that bacteria stimulate haemocytes to extrude intact lysosomes into the haemolymph, a process referred to as degranulation. The resulting release of lysosomal hydrolases is assumed responsible for associated host and non-host tissue damage (Feng 1988, Hose & Martin 1989, Watanabe 1999) and may be one mechanism by which bactericidal activity is seen to rise in lobster haemolymph after inoculation of formalin-killed bacteria (see Sinderman (1971) for review). Recent work on degranulation of human eosinophils has suggested that the eosinophils do not discharge granules to the cell surface (exocytosis) but by lysis (Watanabe 1999), and the process in lobster haemocytes may be the same.

Stress-related opportunistic infections result in an observed reduction in haemocyte numbers presumably by attrition (Stewart *et al.* 1967; Stewart & Rabin 1970; Newman & Feng 1982; Field & Apple 1995; Jussila *et al*. 1998). Low haemocyte counts result in long clotting times (Sinderman 1971). It should, however, be noted that the same effect can occur through an

increase in blood volume, and such changes are seldom measured. Changes in haemocyte counts with moulting may also be volume related (Tsing *et al*. 1989).

Is haemolymph sterile? There is ongoing debate. While many hold that the presence of bacteria in the haemolymph is indicative of septicaemia (Lightner 1977) and is a common result of stress (Lightner 1988), bacteria can be isolated from haemolymph of apparently healthy crustaceans. These include *Procambarus clarkii* (Scott & Thune 1986), *Homarus americanus* (Cornick & Stewart, 1966, 1968), *Callinectes sapidus* (Colwell *et al*. 1975), and *Penaeus vannamei* (Gomez-Gil *et al*. 1998). However, bacterial infection following stress can occur rapidly during capture and transport (Johnson 1976; Messick & Kennedy 1990) making it extremely difficult to ensure that unstressed 'healthy' crustaceans have been sampled. In addition, some bacteria, such as *Aerococcus viridans* var. *homari* appear to be difficult for the host to kill and eliminate (Stewart & Rabin 1970). Lesions associated with foreign body rejection can be used as an indicator of health status (L. Evans, Curtin University, pers. comm).

Cellular defense mechanisms- toxic insults

Toxins come from three main sources - environmental contaminants; toxins associated with foreign invaders (Bowser *et al*. 1981); and toxins resulting from tissue damage and haemocyte degranulation. Reactions to toxins have been studied using injected irritant substances such as turpentine (Fontaine *et al*. 1975). The heart is the organ most affected by circulating turpentine in the haemolymph (Fontaine *et al*. 1975). An acute inflammatory reaction produces melanized hemocytic nodules in the heart followed by influx of haemocytes and fibrocytes. Scar tissue is also formed as numerous collagin like fibres replace myocardial fibres in which numerous melanized nodules are interspersed. The myocarditis reported by Wada *et al*. (1994) in *Panulirus japonicus* may represent the end result of such a toxic insult.

Conclusions

Rapid advances are being made in the understanding of the humoral mechanisms of host defence in decapod crustaceans. However, the histopathology of cellular defense mechanisms, though first studied over 100 years ago, is still poorly studied. The influence of environmental stress, nutritional and moult status of the host on defence responses, all areas of critical importance to animal husbandry and production in aquaculture, have only recently drawn attention (Jussila *et al.,* 1998; Hall & van Dam, 1998) and are difficult to interpret in the absence of a basic understanding of the cellular defense mechanisms. Much remains to be discovered.

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