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State of the art of immunological tools and health control of penaeid shrimp

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Abstract

Shrimp farming constitutes an important source of revenue and employment in many developing countries. However, infectious diseases have affected the profitability of the shrimp industry. For this reason, disease prevention is a priority and shrimp immunology has become a prime area of research. In such a perspective, studies into the value of cellular and humoral parameters as indicators of shrimp condition are being carried out, with the intention of developing criteria for sanitary surveys, immunomodulation studies and selection programs for shrimp with high resistance to pathogens. Several quantitative, fast and easy procedures are being adapted to evaluate the expression of the immune response of shrimp. In regard to cellular parameters, the hemogram and two cellular mechanisms, the radical oxygen intermediates (ROIs) generated during post-phagocytic events and phenoloxidase (PO) activity have been considered as potential markers. Concerning humoral parameters, the antibacterial activity of plasma and the concentration of plasma proteins can be considered as criteria of health status. Information is presented about the immunological tools used to evaluate these health markers and the results concerning the clinical significance of response modifications. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Shrimp farming has had an impressive growth in many developing countries, where this activity has attained a great economic and social importance. For example, in Ecuador, shrimp is the third most important export product and constitutes a significant source of revenue and employment. However, the shrimp industry has always been affected by infectious diseases, mainly of bacterial and viral etiology (Lightner et al.,

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1983; Kroll et al., 1991; Mohney et al., 1994; Hasson et al., 1995; Flegel, 1997), causing great loss of production. The sustainability of the shrimp industry depends largely on disease control and the health status of shrimp. From this point of view, the immune system is a tool to assess shrimp health (Bachère et al., 1995a) and others workers have suggested furthermore the value of immune parameters as biomarkers in ecotoxicology.

For evaluation of cellular and humoral parameters of the immune response of cultured shrimp, the development of simplified procedures has played a vital role for the development of immunoassays. Several authors have been working on the quantification of different cellular and humoral parameters of the immune response of cultivated shrimp species. Among the available tools, we can find hemogram counts (Le Moullac et al., 1997), reactive oxygen intermediates (ROIs) measurement (Song and Hsieh, 1994; Bachère et al., 1995b; Muñoz et al., this issue), phenoloxidase (PO) activity quantification (Hernández-López et al., 1996; Le Moullac et al., 1997), antibacterial activity measurement (Sung et al., 1996), determination of plasma protein concentration, and specific antibodies against several humoral proteins (Rodríguez et al., 1995; Vargas-Albores et al., 1996).

In this review, information is presented regarding immune tools frequently used to evaluate the immune response of penaeid shrimp, their application to identify health markers, and the knowledge acquired on their clinical significance.

2. Hemogram counts

Haemocytes play a central role in crustacean immune defense. Firstly, they remove foreign particles in the hemocoel by phagocytosis, encapsulation and nodular aggregation (Söderhäll and Cerenius, 1992). Secondly, haemocytes take part in wound healing by cellular clumping and initiation of coagulation processes through the release of factors required for plasma gelation (Johansson and Söderhäll, 1989; Omori et al., 1989; Vargas-Albores et al., 1998), and carriage and release of the prophenoloxidase (proPO) system (Johansson and Söderhäll, 1989; Hernández-López et al., 1996). They are also involved in the synthesis and discharge in the haemolymph of important molecules, such as α_2-macroglobulin (α_2M) (Rodríguez et al., 1995; Armstrong et al., 1990), agglutinins (Rodríguez et al., 1995), and antibacterial peptides (Destoumieux et al., 1997; Schnapp et al., 1996; Lester et al., 1997). The hemogram consists of the total haemocyte count (THC) and the differential haemocyte count (DHC). For the DHC, most researchers agree with the identification of three cell types in penaeid shrimp: large granule haemocytes (LGH), small granule haemocytes (SGH) and agranular haemocytes or hyaline cells (HC) (Tsing et al., 1989; Martin and Graves, 1985; Rodríguez et al., 1995; Van de Braak et al., 1996).

For crustaceans, some information exists on the importance of THC in pathogen resistance. Persson et al. (1987) reported in *Pacifastacus leniusculus* a relationship between haemocyte number and its resistance to the parasitic fungus *Aphanomyces astaci*. They demonstrated that a decrease in the haemocyte number of crayfish harbouring *A. astaci* as a latent infection resulted in an acute infection with incomplete melanization of fungus hyphae, leading to the death of the crayfish. Le Moullac et al. (1998) observed that *Penaeus stylirostris* with a low THC due to a hypoxia situation, became more sensitive to infections with highly virulent *Vibrio alginolyticus*. In this study, the DHC was also altered, with a significant decrease in HC and SGH. The physiological importance of the number and composition of haemocytes is suggested by the modifications observed during the moult cycle. In *P. japonicus* (Tsing et al., 1989) and *P. stylirostris* (Le Moullac et al., 1997), the highest haemocyte number was found during the postmoult stage, while the lowest was associated with the intermoult stage. Similar variations were seen in *Sicyonia ingentis* (Hose et al., 1992) in which the most important release of haemocytes from hematopoietic tissue occurs during postmoult stage. As far as DHC is concerned, the highest number of LGH in *P. stylirostris* and *S. ingentis*, occurs in intermoult (Le Moullac et al., 1997; Hose et al.,1992). The HC peaked during the ecdysial period in *S. ingentis* and *P. japonicus* (Hose et al., 1992; Sequeira et al., 1995). The presence of high numbers of HC in the ecdysial period (when the cuticule is weak) seems important, because they initiate the coagulation and also could be involved in cuticle formation (Hose et al., 1992). The high LGH concentration in *P. stylirostris* haemolymph during intermoult could be related to high PO activity and vibriosis resistance (Le Moullac et al., 1997).

THC can be easily determined using a hemocytometer, whereas determination of DHC requires a more complex haemocyte identification. DHC can be determined by the use of morphological criteria such as size and shape of cells and the difference of haemocyte refractivity using a phase contrast microscope (Tsing et al., 1989; Martin and Graves, 1985; Le Moullac et al., 1997). Although this technique is rapid, it should be mentioned that when using this technique it is easy to obtain large variations in results possibly due to interpretation errors.

Different haemocyte types can be determined using cytochemical studies of enzyme activity detection or specific stains. In *S. ingentis*, Hose et al. (1987) reported that acid phosphatase activity was more abundant in SGH, while HC are distinctively stained by Sudan black. In *P. japonicus*, Sequeira et al. (1995) performed cytochemical stains over haemocyte subpopulations separated by flow cytometry, and reported positive peroxidase activity only in LGH. The results obtained from cytochemical stains for penaeid shrimp indicate that these specific stainings can differentiate between the types of haemocytes and provide additional information on their functions. An alternative method for cell identification is the use of monoclonal antibodies (mAbs) in order to find antigenic markers of different cell types. Using mAbs against different subpopulations of haemocytes separated by isopycnic centrifugation on a Percoll gradient, Rodríguez et al. (1995) found in *P. japonicus* that HC share epitopes with SGH, and that an antigen was specifically expressed for LGH. Monoclonal antibodies could be considered as powerful tools for the development of haemocyte lineages and haemocyte proliferation studies, as well as for the isolation and study of plasma components.

3. Measurement of reactive oxygen intermediates (ROIs)

Phagocytosis is the most common reaction of cellular defense. During phagocytosis, particles or microorganisms are internalized into the cell which later forms a digestive vacuole called the phagosome. The elimination of phagocyted particles involves the release of degradative enzymes into the phagosome and the generation of ROIs. This last process is known as the respiratory burst. The first ROI generated during this process is the superoxide anion (O_2^-). Subsequent reactions will produce other ROIs, such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and singlet oxygen ($^1O_2$). Hydrogen peroxide can be converted to hypochlorous acid ($HOCl^-$) via the myeloperoxidase (MPO)–H_2O_2–Cl system, forming a potent antibacterial system (for review see Bayne, 1990; Anderson, 1996).

In penaeid shrimp most studies concerning phagocytosis have been performed through observations of clearance processes of injected bacteria or particulate materials (Fontaine and Lightner, 1974; Tsing, 1987; Martin et al., 1993) but this procedure is inappropriate to quantify phagocytosis. In invertebrates, most of the studies regarding ROIs generation have been performed on molluscs (Dikeboom et al., 1985; Bachère et al., 1991; Pipe, 1992; Noël et al., 1993; Anderson, 1994) and some quantitative procedures have been applied for shrimp research, as the nitro blue tetrazolium (NBT) reduction technique for the measurement of intracellular O_2^- and the reduction of ferricitochrome C for extracellular O_2^-. The determination of H_2O_2 is performed by horseradish peroxidase (HRP)-dependent oxidation of phenol red, while chemiluminescence (CL) is used for the measurement of light emission from ROIs.

The first evidence that crustacean haemocytes produce ROIs was given by Bell and Smith (1993) in the shore crab *Carcinus maenas*. They showed O_2^- generation from hyaline cells using phorbol myristate acetate (PMA) as elicitor. In *P. monodon*, Song and Hsieh (1994) described for the first time the oxidative metabolism in penaeid shrimp. They measured O_2^- using the NBT reduction technique, and H_2O_2 by (HRP)-dependent oxidation of phenol red and detected an MPO-like enzyme activity. Bachère et al. (1995b) demonstrated the existence of respiratory burst in *P. japonicus* induced by PMA and Zymosan, measuring the CL using a scintillation counter. In *P. vannamei*, Muñoz et al. (this issue) worked on a simplified procedure to measure intracellular O_2^- by the NBT reduction assay in microtiter plates. The measurement of the activity in unstimulated haemocytes (base activity) allows the detection of previous excitation states of the haemocytes, that could indicate the existence of an inflammatory process. The assay was found to be specific, obtaining a decrease in O_2^- by SOD and an inhibition by *N*-ethyl-maleimide (NEM).

Despite the limited number of studies focusing on respiratory burst in penaeid shrimp, the actual results are very interesting in view of their value as biomarker of environmental disturbances (see Le Moullac and Haffner, this issue). Furthermore, the importance of respiratory burst as a microbicidal mechanism in penaeid shrimp is strongly suggested by the fact that pathogenic bacteria of shrimp have developed ways of circumventing this mechanism. In *P. vannamei*, O_2^- generation is not produced when virulent *Vibrio vulnificus* is used as elicitor, as opposed to strong stimulation generated by *V. alginolyticus* and other bacteria, such as *Escherichia coli* (Muñoz et al., this issue).

4. Measurement of prophenoloxidase (proPO) and phenoloxidase (PO)

The PO is responsible for the melanization process in arthropods. The PO enzyme results from the activation of the proPO enzyme. The proPO activating system has been very well studied in crustaceans, specially in crayfish, for which some reviews have been written (Söderhäll and Cerenius, 1998; Söderhäll et al., 1996). Melanin and its reactive intermediates have shown to be fungistatic (Söderhäll and Ajaxon, 1982; Persson et al., 1987).

In penaeid shrimp, the first work on melanin formation was descriptive, focusing on histochemical observations of its presence in inflammation sites with hemocytic activity (Lightner and Redman, 1977). Cytochemical stainings of shrimp haemocytes showed that the proPO system was confined to LGH and SGH (Hose et al., 1987; Tsing et al., 1989; Sequeira et al., 1995). The process of activation of the proPO system has been studied in several penaeid shrimp (Hernández-López et al., 1996; Vargas-Albores et al., 1997; Perazzolo and Barraco, 1997; Sung et al., 1998). The release of the proPO system is amplified by peroxinectin, a 76-kDa protein identified in haemocytes. This protein has cell adhesion, degranulation, opsonic and peroxidase activity (Johansson et al., 1995). The proPO has been cloned and sequenced in crayfish (Aspan et al., 1995) and *P. monodon* (Sritunyalucksana et al., 1999).

The PO activity is measured spectrophotometrically by recording the formation of dopachrome from L-dihydrophenylalanine (L-DOPA) at 490 nm (Leonard et al., 1985). PO can be obtained in different ways. The proPO system is released from haemocytes by incubating them with laminarin or zymosan as elicitor in presence of Ca^{2+} (Vargas-Albores et al, 1993a; Le Moullac et al., 1997). PO can be also obtained from cellular lysates containing inactivated proPO system; trypsin is used to activate the proPO to PO (Smith and Söderhäll, 1991). The procedure of PO activity assay has been simplified, the reaction carried out completely in microtiter plates (Hernández-López et al., 1996). The expression of proPO gene can be investigated in haemocytes isolated from shrimp. The amount of the proPO transcripts is evaluated in haemocytes by Northern blot analysis (Lind, personal communication).

Using these different approaches, the function of the proPO system can be better understood in relation to the health status of shrimp. Some studies have shown that proPO could be used as health and environmental markers because changes are correlated with infectious state and environmental variations (Le Moullac and Haffner, this issue) which has recently been confirmed also at the gene expression level (Lind, personal communication).

5. Antibacterial activity quantification

Antibacterial peptides and proteins have been well studied in arthropods, mainly in insects and chelicerata (for reviews see Hetru et al., 1994; Iwanaga et al., 1998), where the families of antimicrobial molecules have been isolated and characterized. In crustacean, some studies have shown the ability of crustacean haemolymph to inhibit bacterial growth (Adams, 1991; Chisholm and Smith, 1992; Noga et al., 1994, 1996). Several antibacterial proteins, active in vitro against Gram-positive and Gram-negative bacteria, were found in *C. maenas* (Schnapp et al., 1996; Smith, 1997). Lester et al. (1997) found a small peptide named callinectin, which was reported to be responsible for the majority of antibacterial activity observed in the haemolymph of blue crab

Callinectes sapidus. Regarding studies in penaeid shrimp, Destoumieux et al. (1997) fully characterized three members of a new family of antimicrobial peptides. These peptides, named penaeidins, are the first antimicrobial molecules found in penaeid shrimp.

In the literature there are reports showing that antibacterial activity in crustaceans can be considered as an environmental marker (Le Moullac and Haffner, this issue). Therefore, many researchers have developed quantitative antibacterial assays based on inhibition of bacterial growth on agar plate (zone inhibition assay and colony-forming units (CFU) inhibition assay) or in liquid medium on microtiter plates (turbidometric assay), to detect the antibacterial ability in crustacean haemolymph. Using the CFU inhibition technique, antibacterial activity has been found in granular haemocytes of the shore crab *C. maenas* and in other crustacean species (Chisholm and Smith, 1992, 1995). Noga et al. (1994) reported a potent antibacterial activity in the serum of *Cal. sapidus*, using the zone inhibition assay and turbidimetric test. Using the CFU inhibition assay, bactericidal activity against Gram negative bacteria have been described in the haemolymph of *P. monodon* (Adams, 1991). In *P. vannamei*, strong antibacterial activity of plasma against different marine bacteria has been observed, using a turbidimetric assay (Rodríguez et al., in preparation). Both techniques (using agar plates or liquid medium) allow the detection of antibacterial activity in crustacean haemolymph. However, from a practical point of view, the second method has the advantage of allowing the analysis of a large number of samples at once.

6. Measurement of plasma protein concentration

Crustaceans have an open circulatory system in which the haemolymph carries out several physiological functions. One of these function is the transport of molecules such as the respiratory protein (hemocyanin) which is the most abundant molecule of the haemolymph (60% to 95 % of total protein) (Djangmah, 1970) followed by the clotting protein and other humoral components. The measurement of plasma protein concentration is based on classic methods, such as the Lowry method (Lowry et al., 1951).

Evidence has been given regarding the physiological importance of the plasma protein concentration and its susceptibility to environmental or physiological changes in the animal. Chisholm and Smith (1994) found a relation between the protein concentration and water temperature, showing low plasma protein concentrations when temperatures are at their lowest and highest in the year. The concentration of total proteins are also related to the moult cycle of the shrimp. In *P. japonicus*, Chen and Cheng (1993) have reported lower levels of protein concentration during postmoult stage (41.37 mg ml^{-1}) as opposed to higher levels (74.90 mg ml^{-1}) found in early premoult (D0). In apparently *P. vannamei* healthy juveniles reared under laboratory conditions the plasma protein concentration is around 120 mg ml^{-1} (unpublished data), but this concentration changes under different environmental conditions. For example, the concentration of plasma proteins is related to the level of protein in the diet (unpublished data). Furthermore, Engel et al. (1993) reported a negative effect of low levels of dissolved oxygen on hemocyanin concentration in serum of the blue crab *Cal. sapidus*.

On other hand several immune molecules have been identified and purified in crustaceans such as the LPS-binding protein (Vargas-Albores et al., 1993b), β-glucan-binding protein (BGBP)(Vargas-Albores et al., 1996,) clotting protein (CP)(Hall et al, 1995; Montano-Perez et al., 1998). In crayfish, some of these proteins are characterized at gene level; BGBP (Cerenius et al., 1994) and CP (Hall et al., 1999) are very similar to their homologues in shrimp. Specific antibodies would allow the use of the ELISA technique to clinically estimate the presence of these proteins in the shrimp plasma. Monoclonal antibodies are available against the clotting factor, α_2M and agglutinin of *P. japonicus* (Rodríguez et al., 1995) as well as polyclonal antibodies against penaeid BGBP (Yepiz-Plascencia et al., 1998).

7. Applications and future research

With currently available knowledge, it is not possible at the moment to define which immune mechanism is the most important for resistance to diseases. For the application of immune criteria in health control and genetic selection programs, it is important to identify disease resistance markers. In such ways, it is important to have experimental infection model (Saulnier et al., this issue) allowing correlation of immune parameters with disease resistance.

To undertake health monitoring on farms, it is necessary to monitor aquacultural performances to know the relationships between environmental conditions and normal or abnormal values of immune responses of shrimp. The use of resistance criteria opens many research possibilities: survivors of experimental infections and shrimp with high level of expression of resistance markers could be used in the selection of broodstock. In addition, it is important to start studies on the heritability of the immune parameters. The application of fundamental knowledge in immunology and other research areas, such as epidemiology and genetics, could contribute to the improvement of management practices in farms and to the domestication of shrimp adapted to rearing conditions.

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