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Knowledge and research prospects in marine mollusc and crustacean immunology

E. Bachère [a,*], E. Mialhe [b], D. Noël [a], V. Boulo [a], A. Morvan [a], J. Rodriguez [c]

[a] IFREMER/CNRS, Université Montpellier 2, UMR 9947, Defense et Résistance chez les Invertébrés Marins, UM2, CP80, Place E. Bataillon, 34095 Montpellier, France

[b] Laboratory of Malaria Research, National Institute of Allergy and Infectious Diseases, NIH, Bethesda MD 20892, USA

[c] CENAIM–ESPOL, PO Box 4519, Guayaquil, Ecuador

Abstract

In the context of infectious diseases in mollusc and shrimp aquaculture, research must be focused on diagnosis for zoosanitary controls but also on obtaining resistant animals. This last strategy depends heavily on the development of knowledge about marine invertebrate immunology. With the establishment of purification protocols for the main invertebrate pathogens, progress has been made in the study of host–pathogen interactions at cellular and molecular levels and in identifying immune effectors involved in the destruction of pathogens. Recent information on molluscs and crustaceans is presented, concerning both hemocyte studies and cellular defence functions and humoral effectors, with special reference to their application to selection of pathogen-resistant animals. With this aim, research prospects will essentially be devoted to the identification and characterization of immune genes, either specific or heterologous, which could be candidates for mollusc and shrimp genetic transformation.

Keywords: Immunology; Molluscs; Crustaceans

1. Introduction

Infectious diseases constitute the main barrier to the development and continuation of mollusc and shrimp aquaculture, each cultivated species being sensitive to several types of pathogen.

In bivalve molluscs, protozoans of the Ascetospora (*Bonamia*, *Marteilia*, *Haplosporidium*) are particularly important, taking into account the extreme commercial losses in

many areas. *Perkinsus* spp. (Apicomplexa) are also well known for their involvement in chronic mortalities and recent epidemics have boosted interest in these pathogens (Goggin and Lester, 1987; McGladdery et al., 1991). Rickettsias and chlamydias are frequently observed in bivalves which has led to considering them as non-pathogenic. However, recent epidemiological surveys have suggested possible involvements in mass mortalities of scallops (Le Gall et al., 1988; Leibovitz, 1989). Several groups of bacteria (*Aeromonas, Pseudomonas, Vibrio* and *Nocardia*) are known to induce mortalities in hatcheries and, to a lesser extent, in growth areas (Paillard et al., 1989). Viruses are still not studied sufficiently in spite of probable high pathogenicity: in 1970, an Iridovirus was suspected of having decimated European stocks of Portuguese oysters, *Crassostrea angulata* (Comps et al., 1976). At present, a Herpes-like virus is associated with larval mortalities in the Japanese oyster *C. gigas* in France (Nicolas et al., 1992) and in New Zealand (Hine et al., 1992).

In shrimps, protozoans, rickettsias and chlamydias have been described (Lightner, 1985) but they do not seem to induce significant mortalities, probably because development of the pathogens is relatively slow compared to shrimp growth. Fungi and bacteria cause severe mortalities in hatcheries. When, as at present, numerous bacterial syndromes are suffered in several areas (Criado-Fornelio et al., 1988; Baticados et al., 1990; De La Pena et al., 1993; Song et al., 1993), the situation becomes critical because regular use of antibiotics leads to resistance problems. As found in insects, the most drastic pathogens are viruses belonging to different groups, in particular baculoviruses, parvoviruses and picornaviruses. Epidemiological surveys of these viruses are still badly performed because of the lack of suitable diagnostic methods but they undoubtedly constitute the main restriction for shrimp production throughout the world.

For many years, research in marine invertebrate pathology was essentially descriptive, focusing on pathogen morphology, anatomopathology and epidemiology. Progress has been made in experimental pathology with the development of several pathogen purification protocols (Mialhe et al., 1985, 1988; Le Gall and Mialhe, 1992). Such protocols are indispensable because of the lack of in vitro systems adapted to cultivation of intracellular Ascetospora pathogens, unlike the Apicomplexa protozoans for which continuous cultures of *P. marinus* have been successfully established in a cell-free culture system (Gauthier and Vasta, 1993; Kleinschuster and Swink, 1993; La Peyre et al., 1993). Besides the preparation of specific molecular probes to diagnose diseases (Mialhe et al., 1992), the availability of purified pathogens has permitted development of in vivo and in vitro models for studying host–pathogen interactions, with a special emphasis on defence processes.

This paper presents information on mollusc and shrimp immunology and future research strategies directed towards obtaining resistant animals.

2. Study of immune system effectors

2.1. Bivalve molluscs

2.1.1. Cellular effectors and immune mechanisms

Hemocytes. In bivalve molluscs, hemocytes constitute mainly the first line of defence against invaders. Agranular hemocytes (hyalinocytes) and granular hemocytes (granulocytes) are classically distinguished and considered by some authors as two distinct cell types (Cheng,

1980). However, hemocytes morphologically constitute very heterogeneous cell populations which are not accurately characterized in terms of cell types and lineages, nor in terms of their respective defence functions. Because of the limitations of morphological features for identifying hemocytes, new approaches have been developed: lectin-binding characteristics (Pipe, 1990a); monoclonal antibodies, previously used to characterize gastropod hemocytes (Yoshino and Granath, 1983; Dikkeboom et al., 1988), were prepared for the Japanese oyster *C. gigas* (Morvan et al., 1991) and for the blue mussel *Mytilus edulis* (Noël et al., 1994). Some monoclonal antibodies were proved to be specific for granular cells and for basophilic granulocytes, respectively in *C. gigas* and *M. edulis*.

The availability of immunological probes will permit an antigenic identification of cell types, particularly useful for determining hemograms with precision. The study of individual hemogram variability will thus be possible in relation to physiological, environmental or stress parameters and in relation to differences in sensitivity to pathogens (Hervio et al., 1995).

Antigenic characterization of hemocytes can be combined with functional studies in order to specify their respective roles in the course of defence response. Furthermore, monoclonal antibodies specific for membrane epitopes constitute unique reagents for hemocyte immunoseparation, for example, using magnetic beads. Finally, monoclonal antibodies, specific for proteins secreted by hemocytes, will also be useful to purify and to characterize these molecules.

In experiments with hemocytes, a routine precaution consists in using an anti-aggregant solution for collecting hemolymph in order to avoid the hemocyte aggregation reaction. A modified Alsever solution was also shown to be efficient in preventing cell degradation and in maintaining hemocytes in a quiescent state. After resuspension in an appropriate medium including calcium and magnesium, hemocytes then recover attachment and spreading behavior, as well as their functionnal capacities. In such experimental conditions, hemocyte quantifications and distributions are reliable. Moreover, living hemocyte subpopulations can be separated by isopycnic centrifugation in isoosmotic media prepared with modified Alsever solution (Bachère et al., 1988).

Phagocytosis. Phagocytosis is considered an important way to control and eliminate foreign particles. This internal defence process is well documented (Bayne, 1990) and classically subdivided into several successive processes:

— *chemotaxis* which is still poorly known in bivalves (Howland and Cheng, 1982)

— *recognition* which is achieved by means of membrane and secreted molecules which are termed opsonins. The identification of lectins in bivalve molluscs and their role as opsonins was demonstrated (Renwrantz and Stahmer, 1983; Vasta et al., 1984). The study of receptors and mechanisms involved in target recognition by hemocytes is of prime importance in understanding subsequent pathogen hemocyte interactions.

— *internalisation* of foreign particles, enclosed in a primary phagosome which then fuses with lysosomes to form phagolysosome. Several lysosomal enzymes have been identified in bivalve hemocytes, among which are acid phosphatase (Yoshino and Cheng, 1976), lysozyme and β-glucuronidase (Cheng et al., 1975; Moore and Lowe, 1977), arylsulphatase, elastase and cathepsin B and G (Pipe, 1990b). These enzymes intervene in the killing

has only been experimentally demonstrated for inducible lysozyme which has a bacteriolytic activity (Cheng et al., 1975).

Bactericidins. Bactericidal activities have been demonstrated in abalones (Cushing et al., 1971) and Japanese oyster *C. gigas* (Mori et al., 1984) but the molecules have never been identified.

Cytolysins. Erythrocytes are agglutinated and lysed in vitro by molecules released by *M. edulis* hemocytes (Leippe and Renwrantz, 1988) but the biochemical nature of these cytotoxic factors remains unknown.

2.1.3. Communication and mediation of defence system

Study of mollusc cytokines was undertaken by referring to the vertebrate mediators which are released by activated immune and non-immune cells and which are able to initiate or regulate host defence responses (Arai et al., 1990). Opioid peptides were demonstrated and involved in communication between *Mytilus* hemocytes (Stefano et al., 1989). Functional studies using mammalian cytokines indicated that *Mytilus* hemocytes were activated. Moreover, using immunological probes specific for IL1 and TNF, cross reactivities were observed by enzymatic immunoassays with mussel hemolymph (Hughes et al., 1990). According to an immunofluorescence assay, hemocytes of *M. edulis*, *O. edulis* and *C. gigas* as well as of the gastropod *Lymnaea stagnalis* showed reactivity with antisera raised against the human cytokines IL1, IL6 and TNF (Adema and Bachère, unpublished results).

2.2. Crustaceans

Knowledge of crustacean immunity is essentially related to the crayfish and *Pacifastacus leniusculus*, some marine decapods such as lobster, *Homarus vulgaris*, and crab, *Carcinus maenas*. Few data concern shrimp species, the species studied most being the ridgeback prawn, *Sicyona ingentis*, the kuruma prawn, *Penaeus japonicus*, and the brown shrimp, *P. californiensis*.

2.2.1. Cellular effectors

Hemocytes. Three types of circulating hemocytes are morphologically recognized in crustaceans (Söderhäll and Smith, 1983; Martin and Graves, 1985; Amirante, 1986; Hose et al., 1987; Tsing et al., 1989) but their actual relationship in terms of lineage remains an open question. The smallest and least numerous hemocytes are the *hyaline cells* which are considered as phagocytes (Söderhäll et al., 1986). The *semigranular cells*, which contain small granules and display some phagocytic capacities, would be specialized in particle encapsulation (Persson et al., 1987). The semigranular cells and the *granular cells* degranulate spontaneously in vitro. These two types of hemocytes would participate in the prophenoloxidase (proPO) system which is an important component of the cellular defence reactions (Söderhäll and Smith, 1983).

As previously established for the crab *C. maenas* (Söderhäll and Smith, 1983), isopycnic centrifugation on Percoll gradient of *P. japonicus* hemocytes permits separation of the three

or extracellularly, to destroy and to

mechanism, since they may act, either intracellularly
digest foreign particles.

Oxidative killing mechanism. In vertebrates, pon
phagocytosis is associated with the productio of re
such as superoxide, hydrogen peroxide, singlt oxy
highly microbicidal (Klebanoff, 1982). This oxidati
in gastropods (Dikkeboom et al., 1985) and inthe s
the production of hydrogen peroxide was demonstr
al., 1985). The technique of chemiluminescene (CL
qualitative and quantitative analyses of the phagocyt
of this activity. The CL corresponds to light emissio
and the generation of ROIs. The CL signals can be an
such as luminol. This phenomenon has been demo
(Larson et al., 1989), *C. gigas* and *Ostrea edulis* (B
edulis (Noël et al., 1993) and bay scallop, *Pecten m*
other hand, phagocytosis has not been related to CL
decussatus, *R. phillipinarum* and *Cerastoderma edul*
data are in accordance with previously established re
uptake in the hemocytes of another clam, *Mercena*
(Cheng, 1976).

CL methodological parameters have been establis
order to obtain a standardized assay. CL is a reliabl
measures. Samples are prepared with identical numbe
either with zymosan particles or soluble stimulat such
which permits comparative analyses. In spite of a re
variability, CL could be adapted for analysing the effe
of any external factor, such as stress, pollutantsor an
et al., 1989; Bachère et al., 1991b).

Oxygen-dependent microbicidal processes were ex
CL activities of oyster hemocyte subpopulations prev
ugation (Bachère et al., 1988). In Japanese oyster,
hyalinocyte phagocytic activity although granulocyte
ticles (Morvan and Bachère, unpublished data). Mo
formed with separated subpopulations of hemocy
eosinophilic granular cells would be the most active
during phagocytosis compared to the basophilic gra
1993).

2.1.2. Humoral effectors

Humoral effectors are soluble factors, some being

Lysosomal enzymes. Lysosomal enzymes originating
extracellular destruction of "invaders" (Cheng and Ro

uitable stimulation of macrophages,
active oxygen intermediates (ROIs),
gen and hydroxyl radical which are
killing mechanism was investigated
allop *Patinopecten yessoensis* where
ted by histochemistry (Nakamura et
) was developed because it provides
sis-related metabolism and a kinetics
resulting from the respiratory burst
plified with an oxidizable compound
strated for the oysters, *C. virginica*
chère et al., 1991a), blue mussel, *M.*
ximus (Le Gall et al., 1991). On the
activity in several clams, *Ruditapes*
(Lopez-Gomez et al., 1994). These
ults on the lack of increased oxygen
ia mercenaria during phagocytosis

ed for marine bivalve hemocytes in
technique for numerous individual
s of hemocytes similarly stimulated
as phorbol myristate acetate (PMA),
atively great individual and species
t on hemocyte phagocytic capacity,
biotics used in aquaculture (Larson

ined more precisely by considering
usly separated by isopycnic centrif-
CL activity was only recorded for
also phagocytosized zymosan par-
over, in *M. edulis*, CL assays per-
s led also to the evidence that
mocytes for the generation of ROIs
ular and hyaline cells (Noël et al.,

oduced by the hemocytes.

om hemocytes could contribute to
ck, 1975). Such a defence function

cell subpopulations, Alsever solution being advantageously used as hemolymph anticoagulant (Rodriguez et al., 1995).

Prophenoloxidase system. Biochemistry of prophenoloxidase (proPO) system activation and regulation is well known in the crayfish (Söderhäll et al., 1990). Briefly, proPO is activated by a prophenoloxidase-activating enzyme (ppA), which is a serine protease previously activated in turn by microbial cell walls. Two protease inhibitors, α2-macroglobulin and a trypsin inhibitor, can block ppA. A protein, 76 kDa cell adhesion factor, released by the hemocytes, amplifies the generation of the proPO system by inducing degranulation of semigranular and granular cells and by stimulating phagocytosis by hyaline cells (review in Johansson and Söderhäll, 1989). About fourteen proteins of the proPO activating system and associated factors in both insects and crustaceans have been purified and characterized (Söderhäll et al., 1990).

Hemocytes and hemolymph proteins of *P. japonicus* have been antigenically characterized with specific monoclonal antibodies. Molecular weights of epitopes were determined by Western-blot or immunoprecipitation (Rodriguez et al., 1995) and some of them were ultrastructurally localised on hemocytes by immunogold electron microscopy. A plasmatic protein of 180 kDa under reduced conditions, reacting with several mAbs, is identified as clotting factor; a protein, with a monomeric form, of 170 kDa is also labelled by specific antibodies in shrimp plasma and localised by immunogold in hyaline cells. Preliminary results of positive cross-reaction with purified crayfish proteins strongly suggest that the monoclonal antibodies are specific for the monomeric form of a shrimp α2-macroglobulin (unpublished results). Moreover, two mAbs identify distinct hemocyte populations separated by isopycnic centrifugation on Percoll gradient. The mAb 40E2 specifically labels granular cells and is specific for a protein of 142 kDa also present in plasma, whereas the mAb 40E10 is the marker for small hyaline and semi-granular cells (Rodriguez et al., 1995). This collection of mAbs is now used for purifying the corresponding proteins which will be characterized and microsequenced. Finally, the mAbs will also be suitable reagents for characterizing the relevant genes by their use as specific probes for screening cDNA libraries cloned in expression vectors.

2.2.2. Humoral effectors

Humoral factors including *agglutinins* or *lectins* have been characterized in various crustaceans: the lobster *H. americanus* (Hall and Rowlands, 1974), the barnacle *Balanus balanoides* (Ogata et al., 1983), the freshwater prawn *Macrobrachium rosenbergii* (Vasta et al., 1983), the crab *C. antennarius* (Ravindranath and Paulson, 1988), the shrimp *P. monodon* (Ratanapo and Chulavatnatol, 1990) and recently in the crayfish *P. leniusculus* (Kopacek et al., 1993). All these lectins in crustaceans are reported to be specific for sialic acid or its derivatives. All the physiologic roles of lectins in invertebrates remain unclear but it appears that these molecules are involved in recognition and defence mechanisms against pathogens and may act as opsonins (in: Vasta, 1990).

The induction of *bactericidins* has been related in lobster hemolymph (*H. americanus*) (Mori and Stewart, 1978) to the response to the injection of different killed bacteria but so far no molecules have been purified and characterized.

Inducible antimicrobial peptides have never been studied in crustaceans. Nevertheless, it must be stated that in other arthropods, and particularly in the chelicerates, antimicrobial molecules named *tachyplesins* and *polyphemusins* have been found in the hemocytes of the horseshoe crabs *T. tridentatus* and *L. polyphemus* respectively (Nakamura et al., 1988; Miyata et al., 1989). These cationic peptides are composed of 17 (tachyplesins) or 18 amino acid residues (polyphemusins), containing two intramolecular disulfide bridges, and with masses of 2263 and 2453, respectively. Chemical synthesis of these peptides was achieved. All the natural and synthetic peptides exhibit almost the same potency in their broad-spectrum antimicrobial activities, namely they inhibit growth of both Gram-positive and Gram-negative bacteria as well as some fungi such as *Candida* and *Cryptococcus* (Akaji et al., 1989; Miyata et al., 1989). In a recent study, tachyplesins and polyphemusins were shown to directly inactivate the vesicular stomatitis virus by destroying its envelope subunits (Murakami et al., 1991).

3. Host–pathogen models

For the purpose of developing research in anti-infectious immunology, in vivo and in vitro host–pathogen models have been established, in particular for bivalve species, because of the availability of pathogen purification protocols (Mialhe et al., 1985, 1988; Le Gall and Mialhe, 1992) and because of progress in hemocyte primary cultures.

3.1. Protozoans

Bonamia ostreae (Ascetospora) is an intrahemocytic parasite of the flat oyster *O. edulis*. Consequently, the interactions of this parasite with host hemocytes have been considered from a pathological and immunological point of view. By using hemocytes from the Japanese oyster, *C. gigas*, interactions can be studied immunologically since this species has been naturally and experimentally proved refractory to *B. ostreae*.

Parasite phagocytosis was first investigated on the basis of experimental infections of hemocyte primary cultures with purified *Bonamia* cells (Mourton et al., 1992). Parasite entry inside hemocytes was then studied using Cytochalasin B as a specific inhibitor of cell cytoskeleton movements. Light and electron microscopic observations indicated that the parasite enters into all the hemocytes, by host-specified phagocytosis, whatever the oyster species (Chagot et al., 1992).

The similarity between *O. edulis* and *C. gigas* hemocyte infection has led to consideration of possible differences in the oxidative killing mechanism. Chemiluminescent assays showed that parasite phagocytosis does not trigger the production of ROIs by hemocytes, neither for *O. edulis* nor for *C. gigas* (Hervio et al., 1989). *B. ostreae* contains large amounts of an acid phosphatase localized in dense bodies of the parasite (Hervio et al., 1991). Such an enzyme is known in some vertebrate parasites as an inhibitor of oxidative killing mechanisms because these acid phosphatases block the generation of superoxide anion (Remaley et al., 1984). To investigate how *B. ostreae* could interfere with the production of oxygen compounds, hemocytes were stimulated with zymosan particles, either before or after adding the parasites. Living or heat-killed parasites were shown to be non-interfering in hemocyte

CL activities. This strongly suggests that *Bonamia* entry into hemocytes is mediated by receptors not involved in the triggering of oxidative metabolism. These results indicate efficient adaptations of the parasite for bypassing the oxidative microbicidal system of the hemocytes. No role has been shown for parasite acid phosphatase.

The respective fates of *B. ostreae* inside hemocytes of sensitive and refractory oyster species has not been determined because of the present difficulties in long-term in vitro culture of mollusc hemocytes.

In Apicomplexa, similar studies have been recently devoted to the effect of *Perkinsus marinus*, an endoparasite of *C. virginica*, on the oxygen-dependent cytotoxicity system of the oyster hemocytes. Whereas in *C. virginica* naturally infected with *P. marinus*, the hemocytes were shown to display increased ROI production as demonstrated by chemiluminescence (Anderson et al., 1992), other recent works suggest that adding *P. marinus* to zymosan-stimulated hemocytes would suppress superoxide ion release (Volety and Chu, 1994).

3.2. Rickettsias

In previous studies, several cases of rickettsiales-like organisms (RLO) have been described in bivalve species (Comps et al., 1977; Buchanan, 1978) and more recently, a branchial RLO infection was associated with mortalities of the sea scallop *Pecten maximus* (Le Gall et al., 1988). The RLO, found in parasitophorous vacuoles of gill endothelial cells, were frequently observed to be free in hemolymph. Therefore, hemocyte–rickettsia interactions were considered with regard to phagocytosis and oxidative killing. An in vitro phagocytosis assay was established with purified RLO (Le Gall and Mialhe, 1992) and primary cultures of scallop hemocytes. Rickettsia phagocytosis by hemocytes was demonstrated by light and electron microscopy, some pictures suggesting an intravacuolar degradation of RLO. However, RLO internalization was not related to the chemiluminescence activity of hemocytes.

Assays with hemocytes, previously incubated with live or killed parasites and then stimulated with zymosan showed a slight inhibition of CL. This inhibitory effect decreased when RLO were previously incubated with L-tartrate, an inhibitor of some acid phosphatases identified in *P. maximus* RLO (Le Gall and Mialhe, 1992). These results would suggest a possible involvement of RLO enzymes in protection against host oxidative killing (Le Gall et al., 1991).

3.3. Viruses

Despite the importance of viruses in marine invertebrate diseases, knowledge about antiviral activities of mollusc and shrimp hemolymph is very limited. This can be partially explained by the lack of cell lines which is an obstacle to the isolation and cultivation of specific viruses. Alternative approaches consist in using in vitro systems with heterologous viruses.

Bivalve molluscs. Experimental in vitro assays were developed using T3 coliphage as a test virus, leading to the identification of a native neutralizing factor in *C. gigas* hemolymph

(Bachère et al., 1990). This factor appeared thermolabile, EDTA-sensitive. Because it can be inhibited by phenylmethylsulphonyl fluoride (PMSF), it has been related to serine proteases.

Similar experiments were performed using lymphocystis disease virus (LDV) (Flugel, 1985) which is morphologically related to the Iridoviruses described in oysters. Preliminary results showed a neutralizing activity of *C. gigas* hemolymph but the factor was not further characterized (Bachère, unpublished data).

Crustaceans. In 1979, such an approach based on heterologous viruses had already been applied (McCumber et al., 1979). In the hemolymph of the blue crab, *C. sapidus*, a neutralizing factor was proved to be active against T2 coliphage but inactive against other coliphages. Further characterization of this factor has not been attempted.

As previously mentioned, tachyplesins and polyphemusins, peptides isolated from horseshoe crabs, display antiviral activities. These molecules are able to inactivate vesicular stomatitis virus by destroying its envelope subunits and they slightly inactivate influenza virus A, whereas herpes simplex virus 1 and 2, adenovirus 1, reovirus 2 and poliovirus 1 are resistant to inactivation.

4. Research strategy and applications

Research in immunology of commercially important marine invertebrates is currently related to infectious pathology but is progressively drawing nearer to genetics, on the one hand to characterize the genes of defence response effectors, and on the other hand to select pathogen-resistant strains, either by quantitative genetics or by genetic transformation (Mialhe et al., 1995). In this context, future research on marine invertebrate immunology would greatly benefit from knowledge acquired concerning vertebrates, invertebrates such as insects, and also plants.

4.1. Specific effectors.

The availability of different in vitro host–pathogen models offers suitable experimental systems to study cellular and humoral effectors.

Cellular responses. In bivalve molluscs, important individual variabilities have been observed in hemograms, hemocyte CL activities and susceptibility to pathogens. For example, experimental infections of flat oysters with purified *B. ostreae* revealed individual variability and strain variability that is expressed in differences in the 50% infectious doses (Hervio et al., 1995). Moreover, a few oysters sometimes survived when exposed to a very high infectious dose. Progeny from such "resistant" oysters are characterized by an increased 50% infectious dose and by an increased number of circulating hemocytes.

In crustaceans, hemocyte data are progressively being acquired and the development of specific probes such as monoclonal antibodies might contribute to a better understanding of cell types and lineage and consequently of hemogram composition.

Because of the economic importance of shrimp aquaculture, the establishment of assays for monitoring the immune states of animals is now a priority. In terms of prophylactic control of infectious diseases, it is necessary to be able to check for all physiological or immunological deficiency. With this aim in view, the recent demonstration of the respiratory burst in crustaceans is particularly important. The production of superoxide ion was shown in the crab *C. maenas* using the method of ferricytochrome-c reduction (Bell and Smith, 1993). The CL technique being used to study the generation of ROIs in the hemocytes of the shrimp *P. japonicus* (Bachère and Rodriguez, in preparation) will now be utilised to test for potentiel immunodeficient compounds, that is, environmental pollutants or antibiotics used in aquaculture.

The study of hemograms, in terms of hemocyte types and numbers, individual and strain variability and microbicidal activities, should be developed. The hemocytic oxidative defence system must be studied further particularly in order to determine its significance against specific pathogens. Other microbicidal mechanisms have to be further investigated, such as the powerful killing system linked to the generation of nitric oxide (Liew and Cox, 1991) which has now been demonstrated in horseshoe crab hemocytes (Radomski et al., 1991) and very recently in two molluscs, *M. edulis* and *Viviparus ater* (Ottaviani et al., 1993). Taking into account possible hemocyte cooperation processes, the investigations on cytokine-like molecules merit particular attention since, in vertebrates, cytokines activate defence cells for killing pathogens (Liew and Cox, 1991)

Humoral responses — inducible antibacterial peptides. Several microbicidal peptide families have biochemically and/or genetically been characterized in insects, showing in some cases similarities with vertebrate molecules. The main insect proteins include: diptericins (Dimarcq et al., 1988), defensins (Lambert et al., 1989); attacins (Hultmark et al., 1983) and cecropins (Hultmark et al., 1982) with an homologous peptide found in intestinal cells of pig (Lee et al., 1989); hemolin, belonging to the immunoglobulin superfamily and thought to initiate an immune response by binding to the bacterial cell wall (Sun et al., 1990). It is also important to consider the tachyplesins and polyphemusins of horseshoe crabs (Nakamura et al., 1988; Miyata et al., 1989) and the magainins isolated from frog skin (Zasloff, 1987). The genes of the majority of these peptides have been cloned. The regulation of their expression and their mode of action are now being investigated, in particular in *Drosophila* with some research based on transgenic animals (Reichart et al., 1992).

Similar to research in insects, it will be advantageous to look for mollusc and shrimp microbicidal proteins using bacterial models since bacteriological techniques are very easy to perform. Demonstration of inducible antibacterial activity can be based on in vitro assays with hemolymph samples to show bacterial killing or growth inhibition. These assays will also be used to follow the proteins in the course of their purification. The ultimate aim will be to clone and to characterize the genes which could be candidates for selecting resistant strains.

Another approach consists in detecting, in mollusc and shrimp genomes, genes which correspond to genes for immunity already characterized in other groups. Such work can be considered since sequences and probes are available with specialized teams. Genes or

portions of genes encoding counterparts of known peptides could be identified from genomic or cDNA libraries either by Polymerase Chain Reaction experiments or by hybridization.

4.2. Heterologous effectors

Heterologous immune effectors have also to be considered because of their potential use in transgenic animals to confer resistance to specific pathogens.

Antibacterial peptides. Production of transgenic plants with insect genes encoding antimicrobial proteins is currently being investigated (Jaynes et al., 1987). Transgenic mosquitos have been produced (Miller et al., 1987) and heterologous immune effectors tested for their parasiticidal effects: synthetic cecropin and magainin were proved to be efficient against oocysts of *Plasmodium* species (Gwadz et al., 1989). A similar approach is being investigated in bivalve molluscs. Thus, using in vitro assay, the cytotoxicity of magainin I has already been demonstrated against *B. ostreae*, the intrahemocytic parasite of *O. edulis*. No physiological damage to the oyster hemocyte was observed (Morvan et al., 1994).

Monoclonal antibodies. Recently, monoclonal antibodies have been successfully tested against the mosquito stage of *Plasmodium* (Warburg et al., 1992). Such results suggest that the expression of immunoglobulin genes in transgenic invertebrates could lead to specific pathogen resistance. This strategy is supported, on the one hand, by work on transgenic mammals and plants with immunoglobulin genes cloned from hybridomas (Storb, 1987; Hiatt and Ma, 1992), and on the other hand by the availability of hybridomas against numerous invertebrate pathogens (Mialhe et al., 1992).

In conclusion, immunology of marine invertebrates is a research priority for aquaculture since infectious diseases are the chief limitation. In fact, the selection of pathogen-resistant strains greatly depends on the identification of immune genes. Progress will also be necessary in experimental pathology to study with precision the effect of these immune genes, and in genetics to have reliable methodologies for mollusc and shrimp genetic transformation (Mialhe et al., 1995).

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Strategy for research and international cooperation in marine invertebrate pathology, immunology and genetics

E. Mialhe [a], E. Bachère [b,*], V. Boulo [b], J.P. Cadoret [b]

[a] *Laboratory of Malaria Research, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892, USA*

[b] *Défense et Résistance chez les Invertébrés Marins (DRIM) IFREMER-CNRS-Université de Montpellier II Case courrier 89, 2, Place Eugène Bataillon, F-34095 Montpellier Cedex 05, France*

Abstract

During the last 10 years, marine invertebrate pathology has moved from morphological description and microscopic diagnosis of pathogens to molecular characterization of these pathogens and probe-based diagnostics. Studies of host–pathogen interactions were undertaken to understand the immunity of molluscs and shrimps with a special new emphasis on immune gene characterization. Recently, genetic transformation has been considered for selecting resistant strains because of the numerous successes obtained with transgenic plants and vertebrates. The production of transgenic molluscs and shrimps, with genes or antisense sequences conferring resistance to specific pathogens, certainly constitutes a new priority for aquaculture. The quick development of research from pathology to immunology and genetics has been made possible partially by developing international cooperation to compensate for the limited manpower, on one hand inside the network of the pathologists, and on the other hand by removing barriers between topics. Regular meetings appear useful for regularly managing research in pathology–immunology–genetics of molluscs and shrimps, for analysing the strategy according to advances in similar fields related to other animal or plant groups, and for improving international cooperation between all scientists concerned from developing and developed countries.

Keywords: Pathology; Immunology; Genetics; Invertebrates

1. Introduction

Marine invertebrate pathology is a young field of research with limited manpower. This apparently difficult situation has in fact contributed to creating a particularly enthusiastic

* Corresponding author.