POLYCHAETES AS ANNELID MODELS TO STUDY ECOIMMUNOLOGY OF MARINE ORGANISMS

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ABSTRACT

Annelids are among the first coelomates and are therefore of special phylogenetic interest. They constitute an important part of the biomass of the seashore, estuaries, fresh water and terrestrial soils. Moreover, they occupy a central position in the trophic networks, as a major food source for fishes, birds and terrestrial fauna. Among Annelids, the large majority of polychaetes is restricted to the marine domain. This report gives an overview of the immune strategies developed by polychaetes to fight pathogens. The potential and interest to use these worms as biomarkers to monitor the influence of environmental perturbation on the immunity of marine organisms is discussed.

I. INTRODUCTION

The field of immunology classically examines the physiological and molecular processes underpinning host defense against pathogens and is usually conducted on laboratory models under optimal conditions i.e. in the absence of natural pathogens. The role and the regulation of immune effector systems under the constraints imposed by life history and ecology came into focus ten years ago. This emerging field called ecological immunology or ecoimmunology is concerned with decorticating the microevolutionary processes that create and maintain variation in immune effector systems and the coordinated host response to pathogens. Immune reactions have been showed to be influenced to various degrees by environmental factors such as anthropogenic pollution.

Invertebrates appear as attractive subjects to study ecoimmunology because of the simple mechanisms underpinning their innate immune systems and their amenability to physiological manipulations in the context of life history and ecology.

Innate immunity which constituted the most ancient first line of immune protection is vital for invertebrate host defense and has become conserved through the animal kingdom. Even if invertebrates lack such critical elements of adaptive immunity as antibodies and lymphocytes, they can resist infections and also transmit lethal diseases to vertebrates, without necessarily being affected. Components that participate in innate immune responses include humoral factors found in body fluids (phenoloxidases, clotting factors, complement factors, lectins, protease inhibitors, antimicrobial peptides…) and cells that kill invading pathogens by using phagocytic or cytotoxic systems.

Annelids belonging to the lophotrochozoan group are primitive coelomates that have developed both cellular and humoral immunity against pathogens. Ringed worms constitute an important part of the biomass of the seashore, estuaries, fresh water and terrestrial soils. Moreover, they occupy a central position in the trophic network, as a major food source for fishes, birds and terrestrial fauna. Among Annelids, the large majority of polychaetes is restricted to the marine domain whereas oligochaetes and leeches can be terrestrial semi or fully aquatic in freshwater or more rarely in seawater.

This report gives an overview of the immune strategies used by polychaetes to fight pathogens. The potential and interest for the use of polychaetes as biomarkers to study the influence of environmental perturbation on the immunity of marine organisms is discussed. Special interest is accorded to species of economic importance and/or used in ecotoxicological studies and bioaccumulation assays.

For understanding immune processes, it is necessary to briefly review Annelids' anatomy. Annelids are characterized by the presence of two compartment containing circulating cells: (i) the blood system with hemocytes, the implication of this compartment in immunity has been studied in leeches only and (ii) the coelom that contains several coelomocytes populations playing functions in immune defense.

1. The Cellular Immune Response of Polychaetes

Cellular immunity includes various mechanisms such as cytotoxicity, phagocytosis and the encapsulation process which leads to the elimination of foreign bodies bigger than
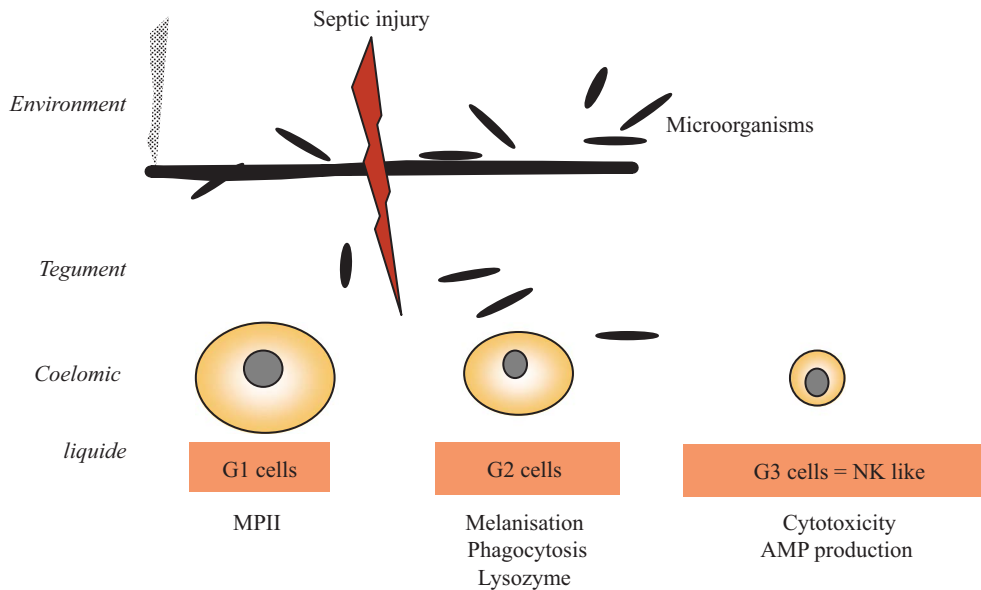
Fig. 1. Hypothetical scheme of the putative immune response of *Hediste diversicolor*. Cellular immunity (melanisation, phagocytosis, cytotoxicity) implicates three types of granulocytes, G1, G2 and G3. Humoral immunity is based on the release from these cells of active substances such as antimicrobial molecules (AMPs, MPII and lysozyme) into the coelomic liquid.

bacteria. As for Arthropods and Molluscs, three major cell types were structurally characterized in the coelomic liquid of Annelids [14]. The clear differentiation in the structure of Annelid coelomocytes is associated with their function: hyaline cells participate in the melanization/encapsulation processes, granular coelomocytes called 'granulocytes' exert phagocytic and cytotoxic activities, eleocytes constitute the functional equivalent of the fat body of insects. The cellular part of the immune system plays a central role in defense against all types of threats. It helps bacterial clearing but also finishes the work in all defense mechanisms (as well as in the current cell) through waste removal. Moreover, it is the sole effective barrier against pathogens of large size, especially parasites, via cytotoxicity and encapsulation. The proportion of cells exerting cytotoxic and/or phagocytic activities is thus an important criterion that can be measured to monitor the impact of environment on individual immunocompetence. Immune cell integrity can also be an interesting parameter. In lungworm, the negative impact of nanoparticles trapped in sediments was evidenced by measuring the lysosomal membrane stability of coelomocytes [13].

In *Hediste diversicolor*, three subtypes of granulocytes were described (Fig. 1), first from ultrastructural observations and later with the aid of monoclonal antibodies [22-24]. Type 1 granulocytes (G1) observed also in Arenicolidae, are large and fusiform cells. Type 2 granulocytes (G2) observed in Arenicolidae, Nereididae, and Terebellidae [8] posses abundant phagocytic vacuoles and are numerically more important than G1. G2 may be related to the hyaline cell type described in leeches, earthworms and non-annelid species of invertebrates. Type 3 granulocytes (G3) are small cells and numerous, with a high nucleoplasmic ratio and a few rounded granules.

To date, G3 have been observed in Nereididae only.

1) Phagocytosis

In polychaetes, active phagocytosis occurs in the battle against bacteria. Experimentally, this process is observed by injecting into the coelomic cavity either small particles or labelled bacteria, which are taken up by endocytosis and stored in the vacuoles of the coelomic cells. This uptake is usually linked with the induction of an oxidative burst of metabolism. Production of highly reactive oxygen radicals represents an effective way of destroying engulfed bacteria. In Nereidae and Arenicolidae, granular coelomocytes through their phagocytic system, are associated with the clearance of bacteria from the coelom [8]. In *H. diversicolor*, after injection of radiolabelled *Vibrio alginolyticus*, a progressive decrease of the radioactivity of the coelomic fluid together with a reduction of the number of granulocytes is observed whereas the number of eleocytes does not change [12]. Contact of isolated coelomocytes with yeast cells show that small foreign bodies are phagocytosed by G2 granulocytes (Fig. 2A), as confirmed by electron microscopy observations [11]. In the free living polychaete *Eurythoe complanata*, the contamination of the environment by copper can be assessed through its immune negative impact measured as phagocytosis impairment, replacing ineffective classical biochemical markers [17].

2) Encapsulation

The encapsulation process has been extensively studied in *H. diversicolor* in an experimental form (implantation of latex beads, [23]) and in a natural context (reactions against different natural coelomic parasites, [23]). Cooperation between
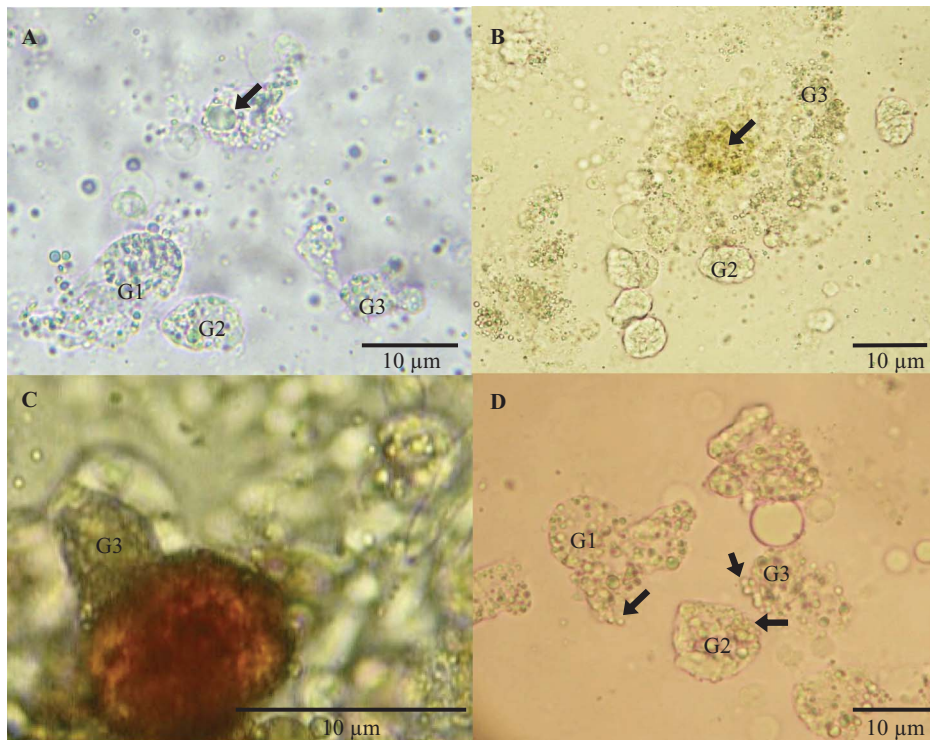
Fig. 2. **Immune functions of isolated *Hediste diversicolor* granulocytes. A- G2 cells are able to engulf foreign particles (here yeast cell pointed by the arrow); B- in the presence of L-DOPA, G2 and G3 cells release compounds that generate melanine (arrow); C- G3 cells exert cytotoxic activity against foreign cells (here coelomocyte from *Eisenia fetida*, colored with neutral red) by tightly adhering to the foreign surface and releasing pore-forming proteins; D- all three types of granulocytes degranulate in presence of bacterial motifs such as LPS (arrows point released granules).**

different coelomocyte populations (except eleocytes) was observed by transmission electron microscopy. Immediately after implantation, G3 cells come into contact with the foreign body and undergo lysis once fixed. The nucleus, cell organites and dense granules are extruded and all form a coating around the latex beads. Several hours later, G2 cells are recruited and form concentric sheets around the implant. Degranulation of the inner sheets of coelomocytes gives rise to a large amount of electron dense fluid of melanic nature (Fig. 2B) [24].

In Polychaetes, encapsulation plays important function in defense against parasites. For example, the development of the coelomic coccidian *Coelotropha durchoni* in *H. diversicolor* constituted an interesting model for analyzing host-parasite interactions [21]. This coccidian develops successfully in its host because of its ability to escape the immune surveillance of the worm. First, it avoids phagocytes by penetrating other cells (eleocytes and muscle cells), where it undergoes a phase of intracellular development. When it goes back to the extracellular compartment, a thick coat protects it from attacks by granulocytes. This coat ruptures to permit fertilization, then a thick cystic envelope forms to protect the fertilized zygotes. Parasites that have lost their coat and remain unfertilized are surrounded with granulocytes and destroyed by encapsulation.

3) Cytotoxicity

The study of graft rejection has highlighted the role of cell cytotoxicity. Studies of graft processing have been extensively investigated in oligochaetes [3, 7]. In polychaetes, observations are relatively scarce and restricted to the family Nereididae. Various organs have been exchanged between individuals of the same *Hediste* species (allograft) or between species (xenograft). Development of the grafts and the host has been followed until normal reproduction of the grafted animals [5]. Autografts are always accepted. Most of the time but not always, allografts and xenografts are rejected, suggesting a mechanism enabling the detection of non-self-tissues. Important differences in the rate of rejection are observed depending on the origin of the grafts. For examples, in the case of xenografts, two distinct responses are observed when *Nereis pelagica* is used as a host: (1) non compatible inter-specific associations: grafts from *H. diversicolor*, *Perinereis cultrifera* and *Platynereis massiliensis* were always rejected and (2) semicompatible interspecific associations, some grafts from *Nereis longissima* or *N. irrorata* were not rejected.

The natural cytotoxic activity of circulating cells was first demonstrated in 1977 in Sipunculoidae, a group related to Annelids [4]. Cytotoxic properties are evaluated by mixing coelomocytes with coelomic cells of another annelid species or with mammal erythrocytes. In *H. diversicolor*, Porchet-Henneré *et al*. showed that after 20 min, G3 bind very tightly to the foreign cells (Fig. 2C) [24]. Many filopodia of G3 cells

extend over and stick to the erythrocytes. Then, the G3 granulocytes release electron-dense granules by exocytosis onto the foreign cells, which finally undergo lysis. Porelike structures are observed on the membrane of lysed cells after they came into contact with coelomic cells, suggesting that a pore-forming protein reminding of the perforin system used by mammal cytotoxic cells, may exist in Nereididae.

2. Humoral Immunity of Polychaetes

In addition to phagocytic, encapsulation and cytotoxic activities, immune cells can secrete antimicrobial substances (antimicrobial peptides (AMPs) and proteins) and other active compounds into the coelomic fluid [26]. Together with other humoral defence molecules, including agglutinins, opsonizing lectins and serine proteases, these contribute to the humoral immunity of both invertebrates and vertebrates. Here, focus is made on AMPs. By contrast with the other listed humoral factors, AMPs produced by polychaetes are generally specific to polychaetes [27]. They are distinct from those produced by the two other classes of annelids that are leeches and oligochaetes and from those produced by all other metazoans. This specificity, added to their potential use as genetic markers (see perspective) to monitor the impact of environmental changes, makes them very attractive to the field of ecoimmunology.

1) Antimicrobial Proteins

The most studied antimicrobial protein in annelids is the lysozyme. This is an enzyme that cleaves the β-1-4 bonds between N acetylglucosamine and N acetylmuramic acid of peptidoglycan. Given the accessibility of the latter in the Gram positive bacterial cell wall, lysozyme is mostly active against this bacterial type. In *H. diversicolor*, lysozym is produced by G1 cells and is detected in the coelomic liquid about 24 h after the worm has been challenged by bacterial injection [9]. The intensity of the response depends on the type of injected bacteria, *Escherichia coli* and *Pseudomonas fluorescent* being the most stimulators. Expression is also induced by bacteria living in the natural environment of the worm (Fig. 3).

Besides lysozyme, a 14 kDa protein sharing a bacteriostatic activity and belonging to the hemerythrin family has been detected in the coelomic fluid of *H. diversicolor* [9]. MPII is a non-hemic-iron oxygen-transport protein acting as an iron scavenger towards bacteria in polychaetes. This protein is expressed by G1 cells, by some muscles and by cells of the intestine [10]. MPII also plays a role of metal binding proteins in toxic metal metabolism in *H. diversicolor* [25]. The participation of MPII in the binding of cadmium appears very effective 48h after exposure and depends on a post-transcriptional mode of regulation [10]. In *Perinereis aibuhitensis* on the contrary, the MPII gene has its expression induced in a time- and dose-dependent manner when exposed to cadmium. Its expression is also modulated by Copper but the effect of cadmium dominates when in combination [29].
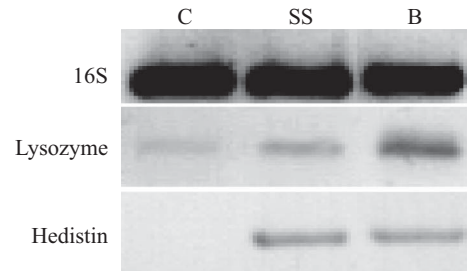

Fig. 3. **Expression of Lysozyme and Hedistin in isolated granulocytes from *Hediste diversicolor* revealed by semi-quantitative RT-PCR. In control animals, lysozyme is weakly expressed in immune cells (C). Four days after a sterile wound, lysozyme expression appears slightly induced (SS), whereas the induction of the gene is clear four days after the injection of heat killed *Vibrio alginolyticus*, a common marine pathogen (B). The same does not apply to hedistin which expression is null in control animals (C), but equally induced after sterile (SS) or septic (B) challenge.**

2) Antimicrobial Peptides AMPs

Numerous studies on the effectors of the innate immune system have demonstrated the contribution of AMPs to the host defense [31]. Antibiotic peptides are small molecules. Based on their structural features, five major classes were defined [6]: 1) linear α helical peptides without cysteines, the prototype of this family are the cecropins; 2) loop forming peptides containing a unique disulfide bond, these were mainly isolated from amphibian skin; 3) open-ended cyclic cysteine-rich peptides among which defensins are the most widespread; 4) linear peptides containing a high proportion of one or two amino acids like indolicidin; 5) peptides derived from larger molecules known to exert multiple functions. In spite of great primary structure diversity, the majority of documented AMPs are characterized by a preponderance of cationic and hydrophobic amino acids. This amphipathic structure allows them to interact with bacterial membrane. AMPs appear to be essential anti-infectious factors that have been conserved during evolution. Meanwhile, their implications in immune processes are different according to species, cells and tissues. The involvement of AMPs in natural resistance to infection is sustained by their strategic location in phagocytes, in body fluids and at epithelial level i.e. at interfaces between organisms and its environment. This action is strengthened by the rapid induction of such AMP genes in bacteria challenged plants or animals [30].

To date, AMPs have been studied in three species of polychaetes, *Perinereis aibuhitensis* [20], *Arenicola marina* [18] and *H. diversicolor* [28]. They all live in estuary sediments rich in microorganisms and toxic agents resulting from pollution. Their abundance in this type of environment suggests that these worms have developed efficient detoxification and immunodefense strategies.

In the Asian clamworm *P. aibuhitensis*, a cationic AMP named perinerin was isolated and partially characterized by Pan *et al*. from the homogenate of adults [20]. This peptide

does not show any similarities with other described AMPs. Perinerin consists of 51 amino acids, among them 4 cysteine residues presumably implicated in two disulfide bridges. Antimicrobial assays performed with the native perinerin evidenced an activity directed against a large spectrum of microorganisms including fungi, Gram positive and negative bacteria at physiological concentrations. The same year, two novel 21 amino acids AMPs, arenicin-1 and arenicin-2, were isolated and fully characterized from the coelomocytes of the lugworm, *Arenicola marina* [1, 18, 19]. Both isoforms were shown to be active against fungi, Gram positive and negative bacteria. Antimicrobial activities of arenicin-1 and 2 appear absolutely equal. Incubation of Gram negative bacteria with arenicin leads to a rapid membrane permeabilization accompanied by peptide intercalation into the bilayer and release of cytoplasm [1]. The isolation of arenicins from coelomocytes lets presume that they may play function in the cellular immunity of *Lumbricus rubellus* in a way comparable to what has been described for hedistin in *H. diversicolor*.

Hedistin was identified from the coelomocytes of the sandworm, *H. diversicolor* [28]. Like perinerin and arenicin, this AMP shows no obvious similarities with other known peptides. Hedistin is active against a large spectrum of Gram positive bacteria. Interestingly, when tested with different Gram negative microorganisms, hedistin is active especially on marine bacteria, *Vibrio alginolyticus*, which is a causative agent of episodes of mass mortality of bivalve larvae in commercial hatcheries. This could be attributable to the capacity of *Vibrio* to extensively degrade native cuticle collagen of *Hediste*. Vibrial collagenase would help bacteria entrance into the worm body, making the mechanical defense barrier of the cuticle inefficient against *Vibrio* invasion. Thus, hedistin synthesis would follow from the adaptation of *Hediste* immune defense towards bacteria of its environment.

The *hedistin* gene is strongly and exclusively expressed in G3 cells [22]. Even if the level of transcript does not increase after bacteria challenge (Fig. 3), "hedistin containing cœlomocytes" appear to accumulate around infection sites where the presence of bacterial motifs triggers the release of the active peptide into the local environment (Fig. 2D). These results are reminiscent of those reported for marine organisms such as the shrimp *Penaeus vannamei* [2], the mussel *Mytilus galloprovincialis* [16] and the horseshoe crab, *Tachyplesus tridentatus* [15]. In *M. galloprovincialis*, microbial challenge provokes the release of the antimicrobial peptide MGD1 from the hemocytes into the plasma. In *T. tridentatus*, bacterial stimulation triggers the degranulation and the release of different immune molecules among them AMPs.

II. CONCLUSION AND PERSPECTIVES

The humoral and cellular immune responses of polychaetes are reminiscent of those reported for other seawater (mussels, shrimps, horseshoe crab….) or terrestrial organisms (earthworm, human…). However, immunity seems to have devel-oped effectors such as AMPs, molecularly adapted to the marine environment of worms. Despite the growing level of interest that the investigation of AMPs has received within marine invertebrates, there is still a scarcity of studies in which these genetic and proteomic tools have been used to investigate the impact of environmental variability on invertebrate immunology. Further investigations will be made in this direction by our group.

Immunity of polychaetes is now relatively well described and may serve as a tool for investigating the impact of stressors on immune cell functionality and consequently on organism's immunocompetence. There are many evidences that anthropogenic pollution influences the immune response in both vertebrates and invertebrates including marine organisms. The immune response to pollutants (heavy metals or pesticides) in the biotope can be quantified and the animals then used as biomarkers to detect and quantify toxics. The immune reactivity tests are often more sensitive than other laboratory methods such as measurement of toxicity, lethality or bioaccumulation since immune activation and possibly disruption of immune functionalities often precede the establishment of the symptoms recorded by the latter methods. Both cellular and humoral immune defense responses under toxic conditions have been investigated in oligochaetes and data clearly demonstrated an alteration of the immunocompetence in exposed animals. To date, this field of research remains poorly explored in polychaetes in spite of an increasing number of studies addressing the impact of environmental stressors on the marine invertebrate immune system. From a general point of view, studying animals from more species and from a greater range of phyla, as well as enlarging these studies to a wider range of stressors, will help further understand the impact of a variable environment on immunity. More specifically, the abundance and large distribution of polychaete species in marine habitats, their key position in the trophic webs and their value as a model for deciphering immune processes in economical species make polychaetes easy models that worth deeper investigations in ecoimmunology.

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