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Review

Molluscan immunorecognition

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Abstract

The immune system in molluscs, as well as in other invertebrates, is endowed with an innate immune response which, however, shows in its recognition processes both molecular mechanisms and effectors similar to those employed by vertebrates in eliminating pathogens and parasites. Furthermore, as in vertebrates, invertebrates also present a profound correlation between immune and neuroendocrine responses. In this review, the players in the immune and neuroendocrine systems have been examined, with particular references to gastropods and bivalves.

Key Words: Molluscs; immune and neuroendocrine responses

Introduction

In the immune system, two categories of immune response are distinguishable, innate or natural and adaptive or acquired. Invertebrates only possess the innate response, which, however, shows in its recognition processes both molecular mechanisms and effectors similar to those employed by vertebrates in eliminating pathogens and parasites (Hoffman ***et al.***, 1999; Medzhitov and Janeway, 2000; Plows ***et al.***, 2005). Furthermore, as in vertebrates, invertebrates also present a profound correlation between immune and neuroendocrine responses (Ottaviani and Franceschi, 1997). Pioneering studies were performed in mammals by Blalock and co-workers (Blalock, 1984; Blalock and Smith, 1985; Weigent and Blalock, 1987, 1989), who have demonstrated that the immune and neuroendocrine systems share common pools of molecules and cooperate in coping with dangerous internal and external agents in order to maintain body homeostasis. Basically, the levels of integration between the two systems can be summarized as follows: i) the use of cytokines and neuropeptides for communication between immune and neuroendocrine systems, i.e. the same signal molecules used to communicate within each system; i) the use of the same cell, the lymphocyte, to perform simultaneous immune and neuroendocrine responses.

This central cell in the immune system has many characteristics of a neuroendocrine cell, e.g. receptors for both hypothalamic releasing factors and neuroendocrine peptides, production of neuro-endocrine hormones and cytokines. Thus the distinction between “hormones”, “neurotransmitters” and “cytokines” becomes open to discussion, as the same molecule can be included in each group depending on the target that is involved. For instance, interleukin (IL)-1 is described as a cytokine when it mediates the interaction between macrophages and T lymphocytes, but it may be considered a neurotransmitter when acts on hypothalamic neurons in inducing fever. In both cases, we have a scenario in which a molecule produced by a cell provokes effects on other close or distant cells, in other words the typical action of a hormone. The distinction, then, would appear to an old conception rather than indicating true functional differences.

The sharing of the same mediators by the immune and neuroendocrine systems suggests that Nature has followed the same general strategy in the construction of these systems. Given this, we have surmized from an evolutionary point of view that both systems have a common origin in which the invertebrate phagocytic immunocyte plays a pivotal role (Ottaviani and Franceschi, 1996, 1997; Ottaviani ***et al.***, 1997a).

This review presents the actors in the immune and neuroendocrine systems and their respective performances in the molluscs, in particular in the gastropods and bivalves.

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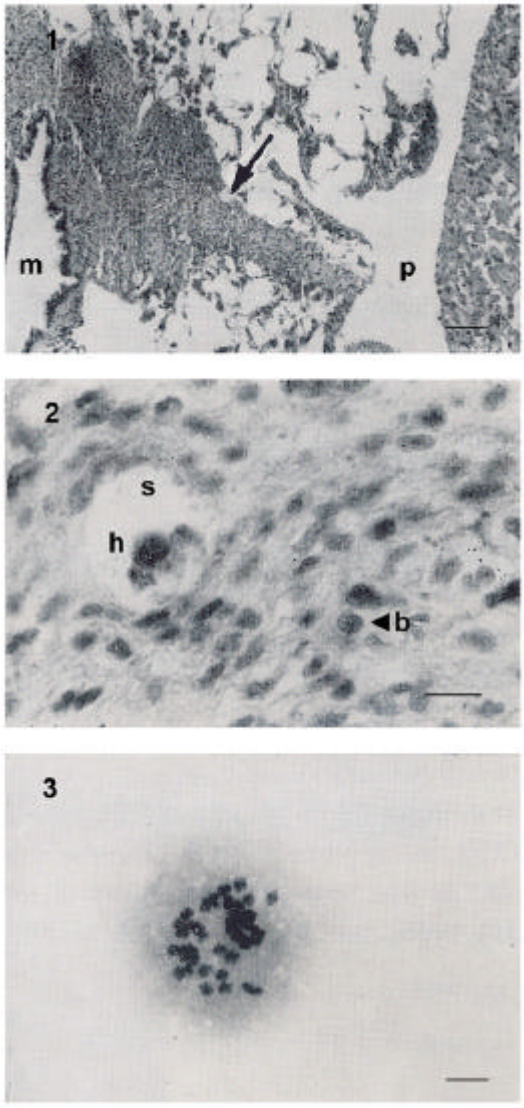
As all invertebrates endowed with a coelomic cavity, molluscs are able to recognize and discriminate between self and not-self principally by means of the cellular and humoral components of the hemolymph. However, other structures, for example the skin and body wall, as well as phagocytic cells located in various tissues are also involved in defense. In gastropods, cells with phagocytic activity are scattered in the connective tissue (*Lymnaea stagnalis*; Sminia *et al.*, 1979) and in organs such as the digestive gland. These cells represent a fixed phagocyte system in *Helix pomatia* (Reade, 1968), while in *Planorbarius corneus* they are distributed throughout the entire gland (Ottaviani, 1990). In *H. pomatia*, antigen-trapping cells have been described in blood sinus and kidney (Renwrantz *et al.*, 1981). It has been reported that while digestible particles are degraded within the immunocytes in the bivalve *Crassostrea virginica* (Tripp, 1958a, b, 1960; Feng, 1959, 1965), the indigestible particles are eliminated via the migration of particle-laden phagocytes across epithelial borders (Tripp, 1960; Feng, 1965).

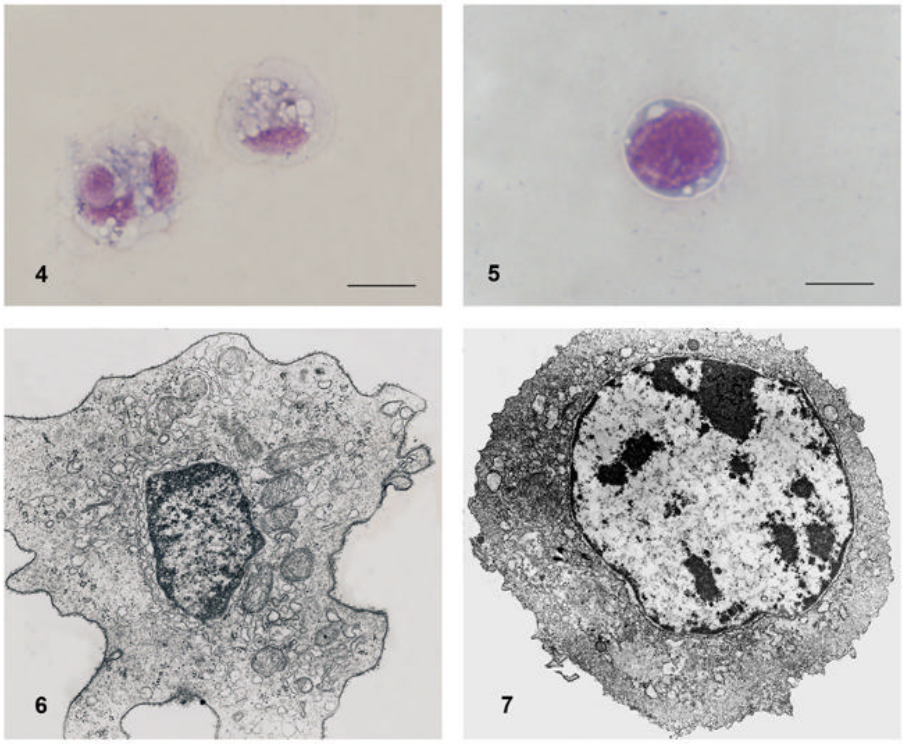
Cellular component

As already reported in insects (Ottaviani, 2005), the classification of the types of immunocytes present in the hemolymph remains an unresolved problem also in molluscs. The cells derive from circulating immunocytes lacking true hemopoietic organs (Sminia, 1981; Ottaviani, 1983). In gastropods, and in particular in various species of Planorbids and *Lymnaea palustris* (Kinoti, 1971; Lie *et al.*, 1975; Rachford, 1976; Jeong *et al.*, 1983; Ottaviani, 1988a), a hemocyte-producing organ (HPO) lying between the mantle cavity and the pericardium has been identified (Figs 1-3). Immunoblasts transformed into immunocytes migrate into the HPO sinus. While no hemopoietic organs have been found in bivalves, it is generally accepted that the immunocytes may originate from connective tissue cells (Cheng, 1981). Together with the number of types of immunocyte, their naming also causes difficulty. Nevertheless, it can be said that the majority of gastropods is endowed with two types of immunocytes. The two types are described in *P. corneus* as spreading and round hemocytes (SH, RH) (Figs 4-7) (Ottaviani, 1983; Ottaviani and Franchini, 1988). The SH show ultrastructural similarities with the spreading amoebocytes of *L. stagnalis* (Stang-Voss, 1970; Sminia, 1972), the granulocytes of *Bulinus guernei* (Krupa *et al.*, 1977) and the granulocytes of *Biomphalaria glabrata* (Harris, 1975; Joky *et al.*, 1983). Despite their different names, these cells show the same morphology and functions, in particular phagocytosis. The *P. corneus* RH is comparable only with the round amoebocytes of *L. stagnalis* (Sminia, 1972, 1981). Cytofluorimetric analysis have revealed that both SH and RH react with several anti-human monoclonal antibodies, including those directed against epitopes typical of mammalian natural killer (NK) cells and cell-adhesion molecules (Table 1) (Franceschi *et al.*, 1991). With regards bivalves, Cheng (1981) in his review proposed dividing the blood cells simply into granular (granulocytes) and agranular (hyalinocytes) types. However, another specialized



Figs 1-3 1) Hemocyte-producing organ (HPO) in *P. corneus* (arrow). m, mantle cavity; p, pericardial cavity. Bar = 50 μm; **2**) Immunoblasts (b) scattered in the stroma and immunocyte (h) in the blood sinus (s) of the HPO. Bar = 10 μm; **Fig 3**) Mitotic division in the circulating immunocyte. Bar = 5 μm (From Ottaviani, 1988a).

type of immunocyte (the serous cell) involved in excretion has been described. In contrast, Mix (1976) suggested that hyalinocytes are a proliferative condition that after various stages mature into granulocytes. In *Mytilus galloprovincialis,* only one cell type in two different stages (young or old) has been proposed (Ottaviani *et al.*, 1998a) (Figs 8-13). These findings support Mix's model, but in *M. galloprovincialis* both young and old immunocytes have the same functions, i.e. phagocytosis and the expression of common signal molecules such as CD5, CD11b and CD16, while the differences in cytology and number seem to be only a consequence of animal aging. It is clear that the classification system is far from unified, as further witnessed by the paper by Hine (1999), who



Figs 4-7 Immunocytes of *P. corneus*. Light microscopy: **4**) spreading (SH); **5**), round (RH) Bar = 10 µm. Electron microscopy: **6**) SH (x18.600); **7**) RH (x11.000) (From Ottaviani and Franchini, 1988) (Reprinted with permission).

suggested a new and more complex scheme than Cheng's (1981) cell-type division.

Humoral component

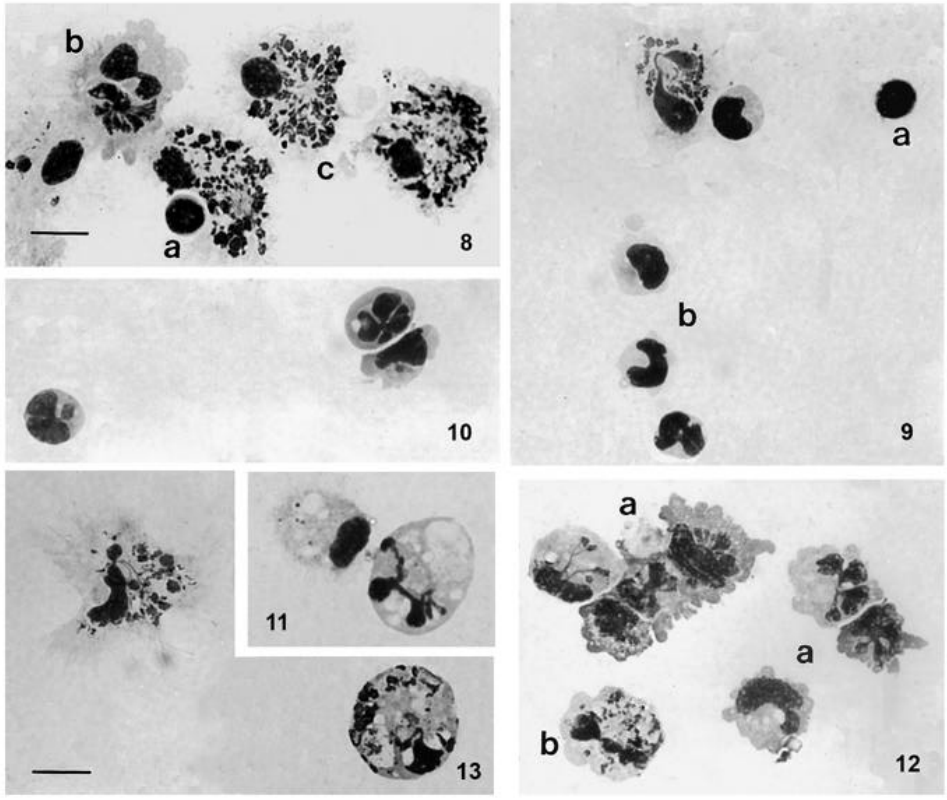
Humoral factors play a fundamental role in the innate immune responses in molluscs. Agglutinins or lectins, bioactive peptides, cytokine-like molecules, nitric oxide (NO), lysozyme and other lysosomal enzymes, anti-microbial peptides and others have been described.

Agglutinins or lectins

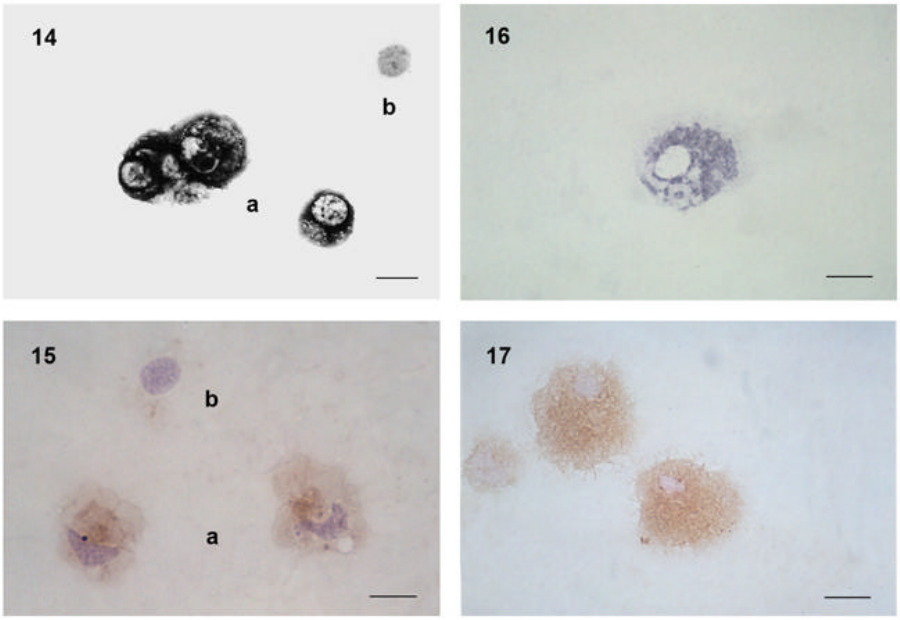
Invertebrates possess humoral components with agglutinating capacity. Agglutinins, or lectins, are glycoproteins consisting of more than one subunit, which function as recognition molecules (Ractliffe *et al.*, 1985; Olafsen, 1986). They may act as opsonins by binding to the non-self material via their carbohydrate recognition sites. However, the binding between the ligand and the phagocytic surface is not been cleared yet. The presence of natural agglutinins or lectins has been documented both in gastropods and bivalves (Ractliffe *et al.*, 1985; Olafsen, 1986, 1996). In gastropods, lectins have been found in *Helix aspersa* (Prowse and Tait, 1969; Hammarström, 1974), *L. stagnalis* (Van der Knaap *et al.*, 1983) and *B. glabrata* (Bretting *et al.*, 1983). In *P. corneus*, natural and induced bacterial agglutinins were isolated by affinity chromatography on Sephadex gel G 150 (Ottaviani and Tarugi, 1986, 1989), and SH are involved in the agglutinin synthesis (Ottaviani, 1988b). Natural agglutinin is a glycoprotein with a molecular weight (MW) of 130 kDa, while the induced form shows a MW of about 330-350 kDa. It is interesting to note that the carbohydrate component of the natural agglutinin contains *N*-acetylmuramic acid, typical of prokariotes, and not sialic acid as expected for eukariotes (Ottaviani *et al.*, 1990a). In bivalves, lectins have been shown in *Crassostrea virginica* (Tripp, 1966), in *Mytilus edulis* (Renwrantz and Stahmer, 1983) and in *C. gigas* (Olafsen *et al.*, 1992). The latter presents lectins able to agglutinate horse and human erythrocytes and bacteria such as *Vibrio anguilarum*. Increased lectin activity was observed after exposing *C. gigas* to *V. anguillarum* for 6 h.

Bioactive peptides

Using a variety of techniques (immuno-cytochemistry, cytofluorimetric analysis, RIA test and *in situ* hybridization), phagocytic immunocytes have been shown to contain a variety of bioactive peptides. In particular, pro-opiomelanocortin (POMC)-mRNA and the related immunoreactive peptides, adrenocorticotropin hormone (ACTH) and β-endorphin, have been detected in the gastropods *P. corneus* and



Figs 8-13 Immunocytes of *M. galloprovincialis*. **8**) Proimmunocyte (a), Type I (b) and Type II (c) immunocytes of an adult specimen. Bar = 10 μm; **9-13**) Different stage of immunocyte maturation from proimmunocyte to Type I and Type II in young specimens: **9**) The cytoplasm of the proimmunocyte (a) gradually increases and the nucleus changes from a round through a reniform (b); **10**) to a polymorphic shape; **11**) vacuola then appear in the cytoplasm; **12**) the cell becomes irregularly shaped (a) and the latter cell type, considered Type I, becomes rich in cytoplasmic inclusions (b) that are typical of Type II; **13**) intermediate forms resembling type II, with a polymorphic nucleus are also found. Bar = 10 μm (From Ottaviani *et al.*, 1998a) (Reprinted with permission).



Figs 14-17 Expression of POMC-mRNA (**14**) and presence of ACTH (**15**) in SH (a) and not in RH (b) of *P. corneus*; expression of POMC-mRNA (**16**) and presence of ACTH (**17**) in *M. galloproviancialis* immunocytes. Bar = 10 μm.

Table 1 Cytofluorimetric analysis of *P. corneus* immunocytes by using mouse anti-human monoclonal antibodies (mAb)

mAb anti-:	SH	RH
CD 1a, CD16, CD26, CD29, CD56	+	+
CD5, CD34, CD45RA, CD54, CD61, CD71	+	-
CD2, CD3, CD4, CD7, CD8, CD11a, CD11b CD11c, CD13, CD18, CD19, CD20, CD21, CD22, CD23, CD25, CD33, CD38, CD43, CD45RO, CD57, HLA-DR, αβTCR, γδTCR	-	-

SH, spreading immunocytes; RH, round immunocytes (Modified from Franceschi *et al.*, 1991)

Table 2 Presence of cytokine-like molecules in molluscs

Species		Refs
Planorbarius corneus	IL-1α,β, IL-2, IL-6, TNF-α, PDGF-AB, TGF-β1	Ottaviani *et al.* (1993b), Franchini *et al.* (1996)
Viviparus ater	IL-1α,β ,IL-2, IL-6, TNF-α, PDGF-AB, TGF-β1	Ottaviani *et al.* (1993b), Franchini *et al.* (1996)
Biomphalaria glabrata	IL-1, TNF-α	Granath *et al.* (1994), Owe-Missi-Oukem-Boyer *et al.* (1994)
Viviparus contectus	PDGF-AB, TGF-β1	Franchini *et al.* (1996)
Lymnaea stagnalis	PDGF-AB, TGF-β1, EGF, neurotrophic factor	Franchini *et al.* (1996), Hermann *et al.* (2000), Fainzilber *et al.* (1996)
Mytilus edulis	IL-1α,β, IL-6, TNF-α	Hughes *et al.* (1990, 1991, 1992), Stefano *et al.* (1991), Paeman *et al.* (1992)
Mytilus galloprovincialis	IL-8, PDGF-AB, TGF-β1	Franchini *et al.* (1996), Ottaviani *et al.* (2000)
Crassostrea gigas	TGF-β	Lelong *et al.* (2000)

Viviparus ater and in the mussel *M. galloprovincialis* (Figs 14-17) (Ottaviani *et al.*, 1990b, 1995a; Franchini *et al.*, 1994). Furthermore, the ACTH receptor-like mRNA has also been detected in the mussel (Ottaviani *et al.*, 1998c). The ACTH, β-endorphin and corticotropin-releasing hormone (CRH) in the immunocytes and cell-free hemolymph were quantified by RIA test (Ottaviani *et al.*, 1990b). With regards CRH receptors, it has been reported that *M. galloprovincialis* immunocytes express molecules homologous to human mRNAs of the two receptor subtypes (CRH-R1 and CRH-R2) (Figs 18, 19) (Malagoli *et al.*, 2000). Finally, a further 14 immunoreactive peptides, including bombesin, CCK-8, neurotensin, oxytocin, substance P and vasopressin, have been detected (Ottaviani and Cossarizza, 1990).

Cytokine-like molecules

Cytokines are soluble factors involved in the immune responses mediating the interactions between different

cell types. However, it has been found that these molecules have a wide range of action as primary mediators of a variety of physiological functions even in non-immune environments such as the neuroendocrine system. Cytokine immunoreactive molecules have been detected in different tissues of various invertebrate species, including molluscs (Ottaviani *et al.*, 2004) (Table 2). Using different technical approaches, these molecules have been detected in immunocytes, in the hemolymph, in eggs, in embryos, in larvae, in neurons and in glial cells from gastropods and bivalves (Hughes *et al.*, 1990, 1991, 1992; Stefano *et al.*, 1991; Paeman *et al.*, 1992; Ottaviani *et al.*, 1993b, 2000; Granath *et al.*, 1994; Owe-Missi-Oukem-Boyer *et al.*, 1994; Fainzilber *et al.*, 1996; Franchini *et al.*, 1996; Lelong *et al.*, 2000). Furthermore, platelet-derived growth factor (PDGF) receptor-α- and -β- and transforming growth factor (TGF)-β receptor (type II)-like molecules have been detected on the plasma membranes of immunocytes from the mussel *M. galloprovincialis* (Kletsas *et al.*, 1998).

Nitric oxide synthase (NOS)

Studies on NOS in invertebrates have been mainly concentrated on the nervous system, and molluscs have been extensively used for investigations of the mechanisms involved in intercellular communication (Stefano and Ottaviani, 2002). With regards the immune system, biochemical, histochemical and immunocytochemical procedures demonstrated the presence of NOS and related immune functions in the immunocytes of *V. ater* (Conte and Ottaviani, 1995; Franchini *et al.*, 1995). These findings are also supported by production of NO by immunocytes from *M. edulis* and *V. ater* (Ottaviani *et al.*, 1993b). NOS was also induced by injection of different cytokines, such as IL-1α, IL-2 and tumour necrosis factor (TNF)-α, in the foot of a mollusc (Ottaviani *et al.*, 1995b).

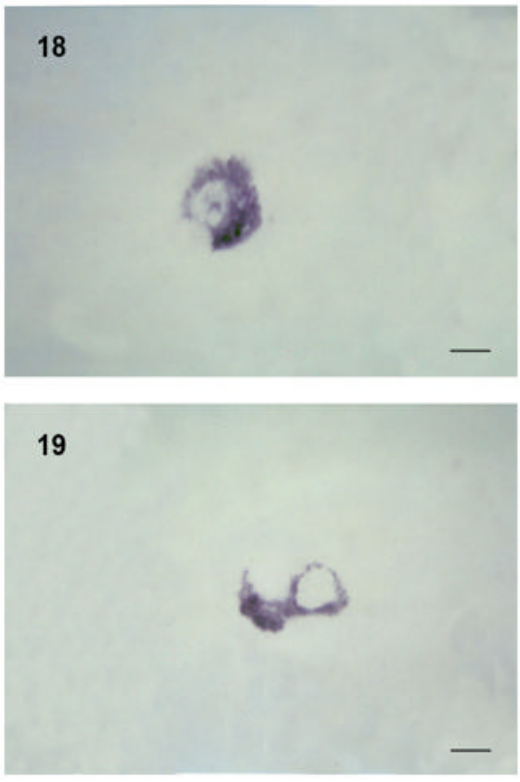
Anti-microbial peptides

Most of the data on anti-microbial peptides regards insects (Bulet *et al.*, 1999), while molluscs have been reviewed only recently (Mitta *et al.*, 2000). In *M. edulis* two defensins (A and B) containing six cysteines and mytilinis (A and B isoforms) have been found (Charlet *et al.*, 1996). *M. galloprovincialis* presents a defensin-like molecule, named MGD1, containing 8 cysteines (Hubert *et al.*, 1996; Mitta *et al.*, 1999), while a second isoform, MGD2, has been identified from immunocyte mRNA (Mitta *et al.*, 1999). Furthermore, mytilinis (B, C, D, G1 isoforms) and myticins (A isoform in the plasma, A and B isoforms in immunocytes) have also been reported (Mitta *et al.*, 1999, 2000).

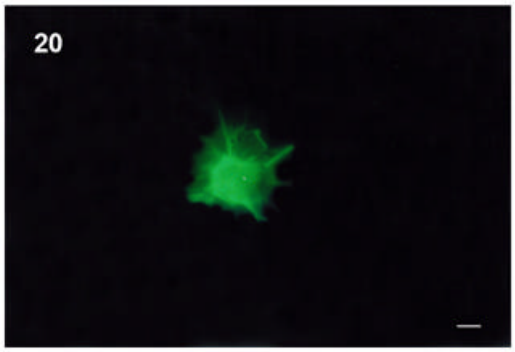
Immune-neuroendocrine interactions

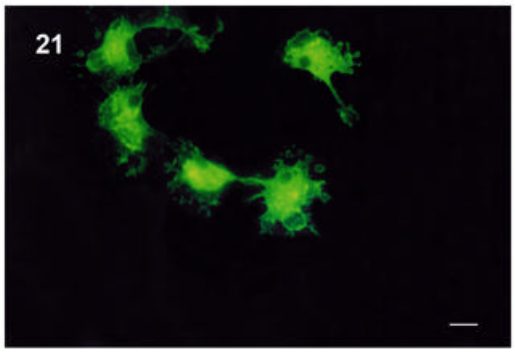
The present section reports how the immune and neuroendocrine responses are intermixed and interconnected in forming an unitarian network to neutralize threatening agents. With regards the immune response, cell shape changes, chemotaxis, phagocytosis, cytotoxicity, encapsulation, trans-plantation and wound healing will be examined, while for the neuroendocrine responses, the stress response is highlighted.

Cell shape changes (the expression of cell motility), chemotaxis (the expression of cell migration) and phagocytosis are the main ancestral mechanisms used by all organisms to eliminate non-self material (Manke and Bade, 1994). Using computer-assisted microscopic analysis, it has been shown that bioactive peptides, such as ACTH and CRH (Sassi *et al.*, 1998; Malagoli *et al.*, 2000), and cytokines, e.g. PDGF, TGF-β and IL-8 (Figs 20, 21) (Kletsas *et al.*, 1998; Ottaviani *et al.*, 2000), provoke changes in the cellular shape of mussel immunocytes. The extracellular signals are transduced by activating the classical pathways, i.e. protein kinase A, C and B. Furthermore, bioactive peptides, cytokines, endorphins and CRH have a chemoattractant effect on molluscan immunocytes (Ottaviani *et al.*, 1997a, b). In particular, ACTH, β-endorphin and their related fragments exert different chemoattractant activity (Ottaviani *et al.*, 1997a). In this context, the first quantitative study on chemotaxis was performed by Schmid (1975) on immunocytes from the snail *Viviparus malleatus* that migrate toward *Staphylococcus aureus*. Chemotaxis has also been observed in the immunocytes of the hard clam *Mercenaria mercenaria* in the presence of bacterial products (Fawcett and Tripp, 1994).



Figs 18, 19 Expression in *M. galloproviancialis* immunocytes of human CRH receptor subtypes: CRH-R1- (**18**) and CRH-R2-mRNAs (**19**). Bar = 10 μm (Modified from Malagoli *et al.*, 2000).





Figs 20, 21 Immunocyte cell shape changes in *M. galloprovincialis* after incubation with IL-8: actin microfilament modifications (**21**). Control (**20**). Bar = 10 μm (Modified from Ottaviani *et al.*, 2000).

Phagocytosis, the last step in the response to non-self particulate material, is also well documented in molluscs (Fig. 22) (Cheng, 1981; Sminia, 1981; Bayne, 1983). Here, it should be underlined that ACTH and its fragments, CRH and cytokines, such as IL-1α, IL-2, TNF-α, IL-8, PDGF and TGF-β all increase phagocytic activity (Ottaviani *et al.*, 1994, 1995b, 2004).

As far as the relation between the NOS system and immunocyte phagocytic activity is concerned, it has been observed that the NO system is able to provoke bacterial clumping and killing, but is not an alternative to phagocytosis, as both are fundamental in bacterial elimination by immunocytes (Ottaviani *et al.*, 1993b; Franchini *et al.*, 1995).

On the whole, these findings suggest that:

i. a direct correlation between chemotaxis and phagocytosis does not exist;
ii. not only peptides with a complete aminoacid sequence, but also peptide fragments of 4 or 5 aminoacids are able to stimulate or inhibit immune functions, as seen with small peptides and mammalian immune functions (Werner *et al.*, 1986);
iii. a close correlation exists between molluscan immune functions and peptides typical of the neuroendocrine system.

Despite the lack of an adaptive immune response, invertebrates are able to cope with pathogen microorganisms present in their environment. Accordingly, primitive but very efficient forms of immunity can be predicted, and cytotoxicity is one of

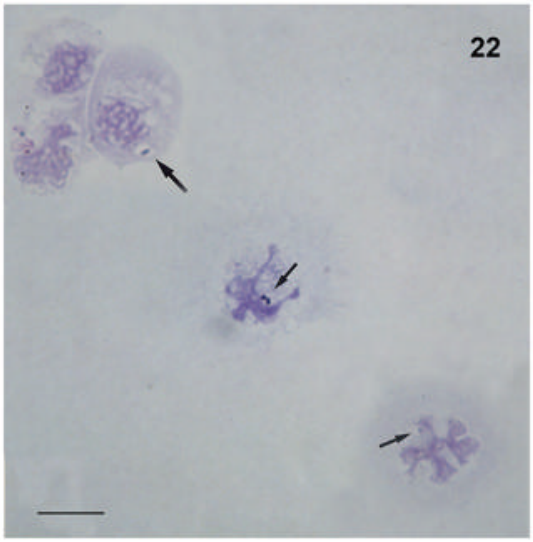
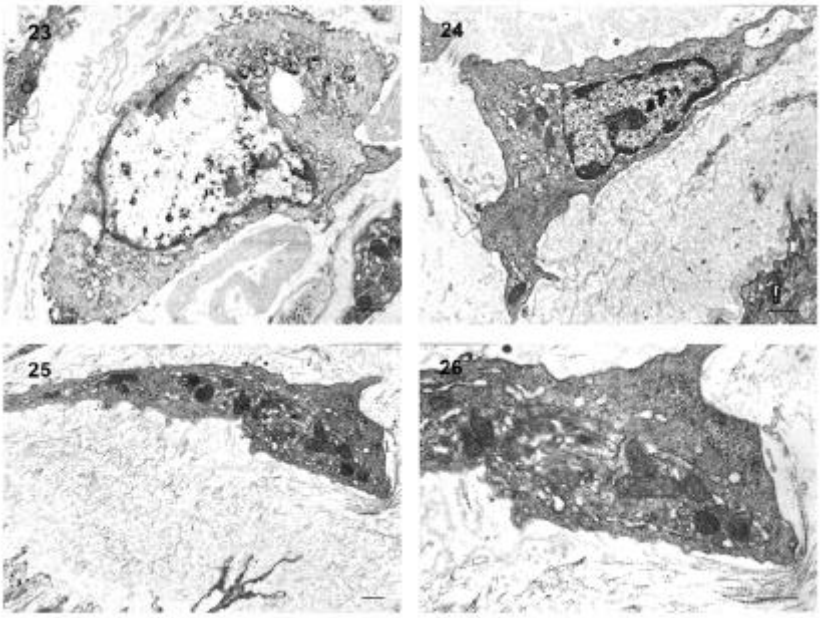


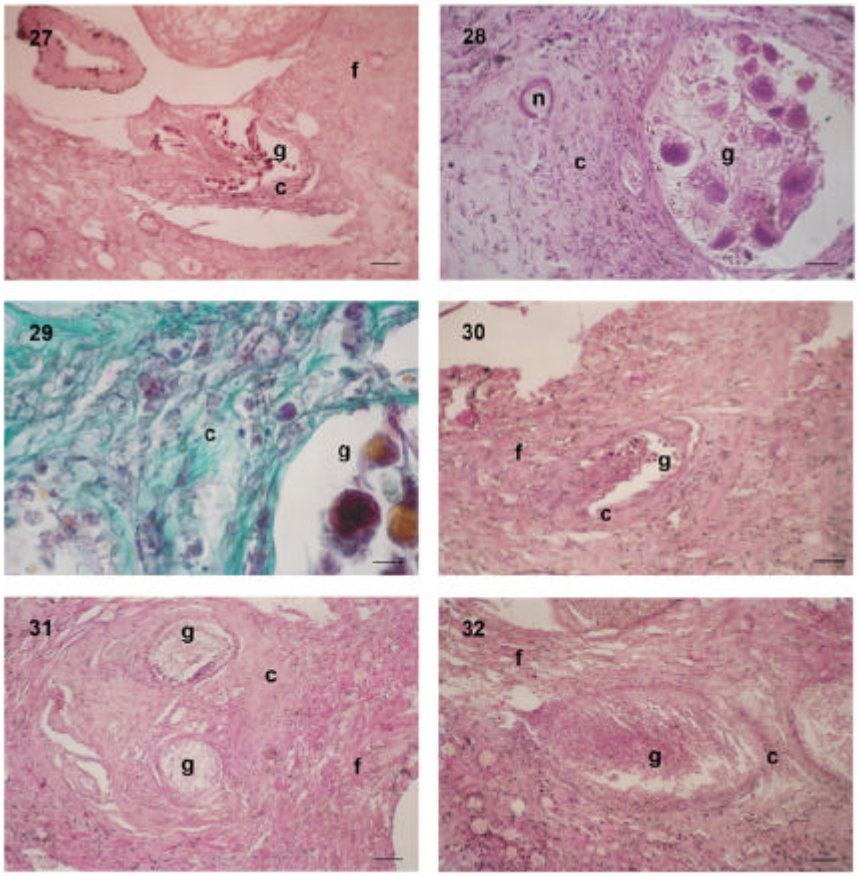
Fig. 22 *M. galloprovincialis* immunocytes phagocitizing bacteria (arrows). Bar = 10 μm.

these. In gastropods, Bayne *et al.* (1980) have found that the immunocytes of *B. glabrata* have a cytotoxic effect on *Schistosoma mansoni* sporocysts. Decker *et al.* (1981) have demonstrated that in different marine invertebrates, including molluscs, cytotoxic activity seems to be independent of prior antigen exposure. In *P. corneus*, only one type of immunocyte, RH, was able to exert cytotoxic activity on K562 cells in a short-term (4 h) ^{51}Cr release essay used to evaluate human NK cell activity. This suggests that a NK-like cytotoxic activity is present in molluscs. Furthermore, the NK-like activity was severely reduced after 18 h incubation at 24 ºC with IL-2 (Franceschi *et al.*, 1991). In the bivalve *M. edulis*, the immunocytes are able to produce cytotoxic substances that cause lysis of human erythrocytes (Wittke and Renwrantz, 1984). The cytotoxic substances, which do not require a preceding contact with target cells (Leippe and Renwrantz, 1988), were purified, found to possess a MW of 72 kDa and act even without free Ca^{2+} (Lieppe, 1989).

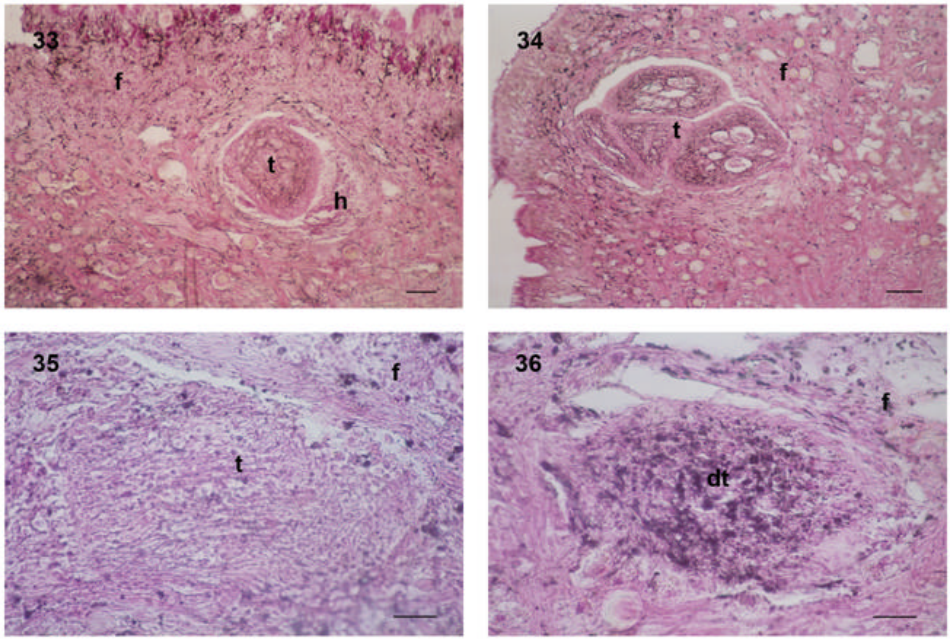
Cytotoxicity molecules may be important in the encapsulation reactions against parasites. For detailed information of the responses of gastropods and bivalves against metazoan, protozoan and fungal parasites, see the interesting chapter in Bayne (1983). The ultrastructural studies of capsule formation in *P. corneus* have shown that both SH and RH are involved (Ottaviani *et al.*, 1991a), and the process can be correlated with the encapsulation process observed in *B. glabrata* using electron microscopy (Harris, 1975) or enzymatic markers such as acid phosphatase (Cheng and Garrabrant, 1977). The granulocyte corresponding to the SH of *P. corneus* (Ottaviani and Franchini, 1988) is one of the two cell types present in *B. glabrata* (Cheng, 1975) responsible for both phagocytosis and the encapsulation process (Cheng and Garrabrant, 1977). The other *P. corneus* cell type, the RH, is the first to reach the graft and behaves in a functionally equivalent manner to *B. glabrata* hyalinocytes (Cheng and Garrabrant, 1977). The *P. corneus* capsule



Figs 23-26 Ultrastructural aspect of the *P. corneus* graft capsule (96 h): RH (**23**); SH (**24**); fibroblast-like cells (**25**, **26**). Bar = 0.5 µm (Modified from Ottaviani *et al.*, 1991a).



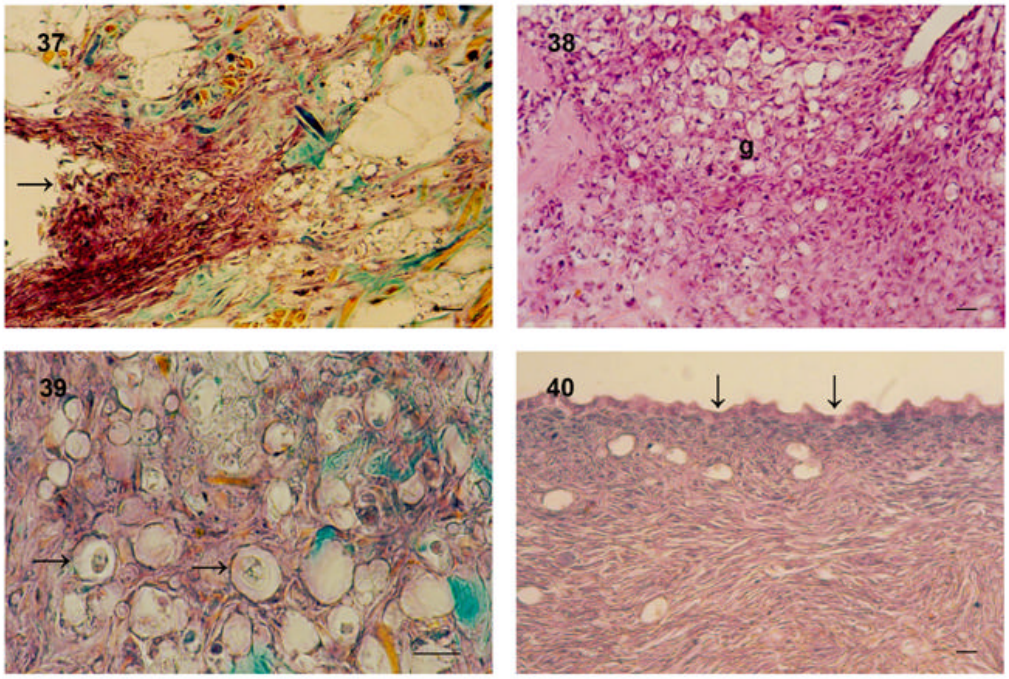
Figs 27-32 Allograft (ganglia) implant in *P. corneus* after 96 h (**27, 28**): the fibres were green with the Gabe and Martoja-Pierson trichromic stain (**29**); 192 h after implant (**30**); xenograft implant after 96 h (**31**) and 192 h (**32**), f, foot; c, capsule; n, new blood vessel; g, ganglia. **27**, **30**, **32**) Bar = 100 µm; **28**) Bar = 30 µm; **29**) Bar = 10 µm (Modified from Ottaviani and Vergine, 1990).



Figs 33-36 Autograft (tentacle) implant in *P. corneus* after 24 h (**33**); 48 h (**34**); 96 (**35**) and 168 h (**36**). f, foot; t, tentacle, h, immunocytes; dt, degenerative tentacle. **33**, **34**) Bar = 100 μm; **35**, **36**) Bar = 30 μm (Modified from Ottaviani and Vergine, 1990).

contains immunocytes and fibroblast-like cells (Figs 23-26), while in *Helisoma duryi normale*, epithelioid cells and multinucleate macrophages have been observed (Cheng and Garrabrant, 1970). In *L. stagnalis*, fibroblast-like cells have been shown to be transformed blood cells (Sminia *et al.*, 1974), and a similar phenomenon probably also occurs in *P. corneus*. Indeed, bearing in mind that *N*-acetylmuramic acid is a SH marker (Ottaviani and Montagnani, 1989), and that both collagen fibres and SH are positive to the corresponding polyclonal antibody, fibres may be formed by the SH that can so be considered fibroblast-like cells (Ottaviani *et al.*, 1991a).

Studies on cellular encapsulation have shown that the extension and the speed of the process is related to the degree of compatibility between the host and the encapsulated parasite from a foreign body (Sminia, 1981). In this respect, transplantation experiments are a good tool to investigate the degree of discrimination of the molluscan recognition system. However, the various gastropod models have not given a uniform response. *Australorbis glabratus* (Tripp, 1961) and *L. stagnalis* (Sminia *et al.*, 1974) are able to discriminate between autografts and xenografts, but fail to recognize allografts. Also in *B. glabrata,* several alloimplats are not rejected (Sullivan *et al.*, 1998). In contrast, *H. duryi normale* (Cheng and Garrabrant, 1970) and *P. corneus* (Ottaviani and Vergine, 1990) recognize allografts. In *P. corneus*, cerebral ganglia from samples of the same (allografts) or different (xenografts) species were implanted in the foot. For the autograft experiments a removed tentacle was used. The histological observations 72 h after alloimplant showed that the immunocytes were stratified, flattened and infiltrated in the graft tissue. After 96 h, the graft was completely encapsulated (Fig. 27). The capsule showed immunocytes, thin fibril bundles loosely dispersed in an amorphous substance and the presence of new blood vessels (Fig. 28), as witnessed by the presence of an angiogenesis process. The amorphous substance and the fibrillar component were positive to bromophenol blue, PAS and alcian blue pH 2.5 reactions. The fibres were green with the Gabe and Martoja-Pierson trichromic stain (Fig. 29). From 168 h onwards, the capsule decreased in thickness. The implanted ganglia showed suffering and after 192 h almost all the structure had been destroyed (Fig. 30). The ganglia xenoimplant in the foot of *P. corneus* was encapsulated after 48 h, and the capsule showed maximum thickness after 96 h (Fig. 31), diminishing drastically after 192 h (Fig. 32). The autograft experiments have revealed that the immunocytes migrated towards the implanted tentacle (Fig. 33), and that after 48 h they were not longer observed (Fig. 34). Later at 96 h, the autograft seemed to integrate into the host tissue (Fig. 35). At 168 h, the degenerative process was observed in the graft (Fig. 36). The non-survival of the autograft can have several explanations, including blood supply, the nature and the number of cells, tissue mitotic activity after vascolarization, the capacity of the graft to stimulate host fibroblasts and so on. This experiment has shown that *P. corneus* seems to have a specific immunorecognition system able to discriminate between the different grafts. In bivalves, allotoimplants were performed by inserting small portions of living mantle from *C. gigas* into a slit in the connective tissue near the palps of another animal (Des Voigne and Sparks, 1969). The findings have indicated that some alloimplants are rejected, while others seem to remain normal during the duration of the experiment. Xenoimplants, performed by placing *Mya arenaria* mantle orthotopically on *M. californianus* mantle (Bayne *et al.*, 1979), have shown that the second- and



Figs 37-40 Wound healing in the gastropod *L. maximus*. **37**). A large number of immunocytes (arrow) stratify at the wound margins; **38, 39**) A well-developed granulation tissue (g) with hematic lacunae (arrows); **40**) The repairing wound is covered by cubic epithelial cells (arrows). Bar = 10 μm (Modified from Franchini and Ottaviani, 2000).

are rejected more rapidly than the first-set, while there is a more localized host response to the third-set implant. However, the Authors have claimed that the response is not specific.

Wound healing is a complex process involving different biological events to restore tissue integrity and functions. This process is characterized by inflammation, new tissue formation and tissue remodelling. Experiments performed on the gastropod *Limax maximus* (Franchini and Ottaviani, 2000), in which longitudinal incisions were made through the skin of the latero-dorsal part of the body, have shown that wound repair is first characterized by an infiltration phase, during which the immunocytes are recruited, accumulated and activated in the injured area (Fig. 37). Flattened immunocytes are stratified at wound margins and actively phagocytize cell debris and damaged tissue surrounding the area. The inflammation response is also observed in other molluscs (Cheng and Garrabrant, 1970; Sminia, 1981; Ottaviani and Vergine, 1990). As in mammals, the second step in the process involves the formation of granulation tissue, in which several small blood lacunae are formed and a provisional matrix is synthesized and deposited by immunocytes presenting fibroblast-like activity (Figs 38, 39). Finally, there is a wound re-epithelialization phase (Fig. 40). Exogenous administration of PDGF-AB and TGF-β1 stimulates the tissue healing process by accelerating all the activities involved. Wound healing experiments in *C. gigas* have shown a similar chronology. Initially, the leukocytes infiltrate, elongate and arrange themselves parallel to the direction of the lesion. Just when the cells have filled the lesion, the scar is formed and subsequently substituted by fusiform leukocytes that create a pseudoepithelium at the body surface. The oyster healing process is closely to that observed in annelids (Des Voigne and Sparks, 1968). The wound healing observed in the freshwater mussel *Anodonta oregonensis* have shown an immediate inflammation response, followed by the repair process, as seen in most vertebrates and other invertebrates. However, there is no pronounced cellular response in this mussel (Pauley and Heaton, 1969).

Memory-type response

The presence of some kind of memory in invertebrates is still debated. The evidence in favour of the existence of a memory-type response in lower and advanced invertebrates has been proposed by various authors (Cooper, 1969, 1976; Hostetter and Cooper, 1973; Karp and Hildemann, 1976; Hildemann *et al.*, 1977, 1979a,b) and by Karp and Rheins (1980). In the present review, a further example of a memory-type response in an invertebrate (*P. corneus*) is presented. According to Hildemann *et al.* (1979), three functional components must be identified as minimal criteria for immunological competence, i.e. cytotoxicity, specificity and memory. Data in favour of the presence of cytotoxicity and specificity have been reported before, while results supporting memory in *P. corneus* have been obtained from humoral and cellular experiments as well as bacterial clearance studies (Ottaviani *et al.*, 1986; Ottaviani, 1992). After injecting the bacteria *S. aureus* and *Escherichia coli* repeatedly into the foot of the mollusc, specific and aspecific agglutinins which were undetectable before injection were observed.

Specific agglutinins observed in direct-agglutination tests showed an increased titre after the second injection. Aspecific agglutinins evaluated in cross-agglutination tests showed no changes in titre after the second injection (Ottaviani, 1992). The *in vitro* bacterial phagocytosis experiments have shown a higher bacterial elimination across the entire time-range considered (30, 60, 90, 120, 150 min) in the snails that had already contacted the bacteria to be phagocytized. Differences between control and sham-injected control snails were not observed (Ottaviani, 1992). The bacterial clearance investigations have revealed that after the second (14 days) and third (73 days) bacterial injections, clearance rates are faster and clearance patterns markedly different than after the first injection (Ottaviani *et al*., 1986).

On the basis of the above findings, some considerations can be advanced. According to Klein (1989), the evolutionary key opening the door to the vertebrate immune system should not be sought in the invertebrate immune system, since the vertebrates are not the crowing phase of invertebrate evolution. Looking at the problem from another point of view, i.e. considering the DNA as the possible prime mover of evolution, this statement is not completely true. The DNA is conservative, but all forms of life are the expression of casual combinations of DNA sequences. The resulting products, that are the expression of selective advantage, appear to be re-proposed every time that new forms derive from DNA. A typical example is represented by molecules and functions of the immune system, such as bioactive peptides, CD, phagocytosis, NK activity and others. In this respect, it is useful to examine the immune mechanisms present and conserved in lower forms in order to understand the complexity of higher life. Furthermore, as every form of life survives, it must possess an immune system sufficient to its needs and, as suggested by Hildemann (1974), it is reasonable to recognize different levels of immunity. Therefore, observations on the structural simplicity of invertebrates and the consequent lack of a true immune system with an anamnestic-accelerated secondary response (Klein, 1989) are quite inadequate, as invertebrates possess immunological competence. However, bearing in mind that invertebrate memory is not based on clonal selection, it would be better to use the term "invertebrate memory", rather than "immunological memory".

Stress response

POMC-derived peptides and CRH are the central mediators of stress response. In mammals, the main circuit involves a hypothalamic-pituitary-adrenal gland axis. CRH produced in the hypothalamic paraventricular nuclei controls the pituitary secretion of ACTH that, in turn, stimulates the synthesis and the release of adrenocorticosteroids. Together with neural activation, glucocorticoids regulate catecholamine biosynthesis in adrenal medulla. The question of the evolution of this apparently very complex type of response that involves several distant organs and a variety of cell types has been resolved in invertebrates by a simplified scheme, in which the basic mechanisms are well conserved. Indeed, the phagocytic immunocytes contain the key mediator molecules and the series follows the same order and pattern, i.e. CRH → ACTH → biogenic amines (BA) (Ottaviani and Franceschi, 1996). In molluscs, in addition to this ACTH-mediated pathway, another more direct CRH → BA pathway appears to exist. As far as BA are concerned, epinephrine, norepinephrine, dopamine have been found in molluscan brain, hemolymph and immunocytes using a HPLC procedure (Ottaviani and Franceschi, 1996). Furthermore, enzymes of the BA biosynthesis and cortisol have been reported in the immunocytes by immunocytochemical procedure (Ottaviani *et al*., 1993a, 1998b; Ottaviani and Franceschi, 1996). Studies on stress have also been performed by Stefano's group. The immunocytes of *M. edulis* produce and reacte to opioid peptides (Stefano *et al*., 1990) and the cellular response to stress observed is the same as that found in mammalian immunocytes. Similarly to vertebrates, cytokines are also involved in molluscan stress responses. Indeed it has been observed that IL-2, IL-1α, and β, TNF-α and β, PDGF-AB and TGF-β1 induce BA release from immunocytes (Ottaviani *et al*., 2004).

All these events occur in the immunocyte, a cell type that has been proposed as an "immune-mobile brain", capable of both immune and neuroendocrine responses (Ottaviani *et al*., 1991a, 1993a).

Concluding remarks

In conclusion, the findings reported in this review support the following considerations and speculations:

- Despite their apparent simplicity, molluscs, as all celomatic invertebrates, are capable of very sophisticated performances regarding immune and neuroendocrine functions;
- Most of the molecules used to perform immune and neuroendocrine responses are well-conserved from invertebrates to vertebrates. In the higher forms of life, their function remains basically similar. It appears that Nature has made new use of old molecules;
- Immune and neuroendocrine functions partially overlap and in some cases they are performed by the same cell, i.e. the immunocyte.

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