ISJ 7: 11-21, 2010 ISSN 1824-307X

REVIEW

Signaling molecules involved in immune responses in mussels

S Koutsogiannaki, M Kaloyianni

Laboratory of Animal Physiology, Zoology Department, School of Biology, Faculty of Science, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

Accepted December 22, 2009

Abstract

Immune system of molluscs is constituted by hemocytes and humoral factors that cooperate for the protection of the organism, triggering a wide range of immune responses. In molluscs, immune responses include phagocytosis, encapsulation, respiratory burst leading to reactive oxygen species (ROS) production and nitric oxide (NO) synthesis, release of antimicrobial molecules and the activation of phenoloxidase system. These responses are mediated firstly by a variety of hemocyte receptors binding to ligands that results to a cascade of signaling events. The processes of hemocytes adhesion to and migration through extracellular matrix (ECM) proteins play a crucial role in cell immunity. Results suggest that cadmium and oxidants induce adhesion to and migration through ECM proteins in *Mytilus gallorovincialis* hemocytes with the involvement of Na^+/H^+ exchanger (NHE), phosphatidylinositol-3 kinase (PI-3K), protein kinase C (PKC), NADPH oxidase, ROS and NO as well as with α2 integrin subunit. Furthermore, the data so far suggests the involvement of additional signaling molecules such as mitogen-activated protein kinases (MAPKs), signal transducers and activators of transcription (STATs), c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), cyclic adenosine monophosphate (cAMP), responsive element binding protein (CREB) and nuclear factor kappa B (NF-kB) in molluscs immunity. Further research in mollusc immune system may lead to a more sufficient protection and to a better control of these economically important organisms.

Key Words: *Mytilus galloprovincialis*; immune response; adhesion; migration; integrin

Introduction

Immune responses are highly complex including a variety of different cellular and molecular processes. The study of the immune processes in invertebrates is of great importance from ecological, economic and public health points of view (Peteiro *et al*., 2007). Furthermore, the study of the immune mechanisms in molluscs is also significant due to their susceptibility to infection by bacteria, viruses, and parasites that makes them transmitters of many diseases affecting different vertebrate species (Barcia and Ramos-Martinez, 2008). However, the available data on the immune responses in invertebrates should be implemented (Humphries and Yoshino, 2003; Tiscar and Mosca, 2004; Canesi *et al*., 2006; Mydlarz *et al*., 2006; Ottaviani, 2006; Barcia and Ramos-Martinez, 2008). In this review we will refer to the phagocytic behavior of *M. galloprovincialis* hemocytes.

Corresponding author:
Martha Kaloyianni
Zoology Department, Aristotle University of Thessaloniki
54124 Thessaloniki, Greece
E-mail: kaloyian@bio.auth.gr

Immune system of molluscs

Among invertebrates, molluscs represent the widest phylum after arthropods. They are considered as excellent bio-indicators and they have been used intensively in research studies. The defense mechanisms of molluscs consist firstly of chemicophysical barriers (external skeletons, cockles, cuticles, mucus) that prevent host invasion and secondly of the circulating hemocytes and humoral factors that operate in co-ordination triggering a wide range of immune responses (Renwrantz, 1990; Rinkevich and Muller, 1996; Hine, 1999). According to Mydlarz *et al*. (2006) the three essential components of innate immunity in invertebrates are: 1) phagocytosis which represents the cell-mediated immunity, 2) activation of humoral responses that result to opsonization, coagulation and melanization and 3) the production of humoral antimicrobial components.

M. galloprovincialis immune system consists in at least four subtypes of hemocytes charged with different tasks in host defense: large granular (R1), large semigranular (R2), small semigranular

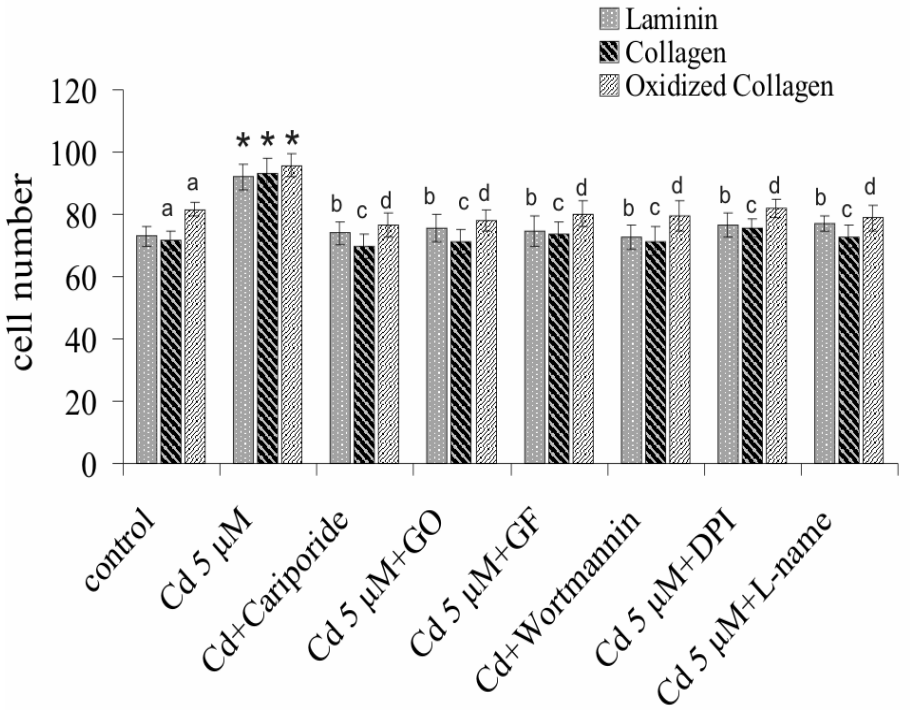

Fig. 1 Adhesion of *Mytilus galloprovincialis* hemocytes to laminin, collagen IV and oxidized collagen IV after treatment with cadmium and inhibitors of NHE, PKC, PI-3K, NADPH oxidase and NO synthase. Hemocytes were pre-incubated with the inhibitors cariporide (20 nM), GO6976 (500 nM) and GF109203X (10 μM), Wortmannin (50 nM), DPI (10 μM) and L-name (10 μM) for 15 min at 20 ^{0}C, $CdCl_2$ (5 μM) was then added and the samples were incubated for 30 min at 20 ^{0}C. The results show the means of at least 4 experiments ± SD. The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-hoc test ($p<0.05$). * indicates significant difference of the sample value with the control value. Values that share ^{a} are significant different between each other. ^{b,c,d} indicate significant difference between each sample value with the respective control value (Cd alone) (Koutsogiannaki, 2008)

(R3), and small hyaline (R4) hemocytes (Garcia-Garcia *et al*., 2008). While large granular (R1), large semigranular (R2), and small semigranular (R3) cells are thought to be phagocytic, and capable of activating the respiratory burst, small hyalinocytes (R4) lack these two capabilities. Nevertheless, all hemocyte subpopulations seem to be capable of nitric oxide (NO) production (Garcia-Garcia *et al*., 2008). The presence of many hydrolytic enzymes in the large granules indicates their connection with lysosomes (Pipe, 1990). Ottaviani *et al*. (1998a) proposed just one type of immunocytes in different stages (young and old) in *M. galloprovincialis* supporting Mix's (1976) suggestion that hyalinocytes (agranular type) are a proliferative condition that after various stages mature into granulocytes. Two of these four subtypes, large granular and large semigranular cells share common features with the mammalian professional phagocytes (Garcia-Garcia *et al*., 2008). Furthermore, hemocytes secrete humoral factors that play a fundamental role in the innate immune responses in molluscs including lysosomal enzymes, agglutinins or lectins, cytokine-like molecules, bioactive peptides, NO, and antimicrobial peptides (Ottaviani, 2006). In addition to these, the defense mechanisms of mussels include the activation of phenoloxidase system (Little *et al*., 2005). Hemocytes are also involved in detoxification through accumulation of metallic and organic xenobiotics in their well developed lysosomal system (Cajaraville *et al*., 1995).

Phagocytosis represents the main cell-mediated immune response and is mediated by the hemocytes. It is comprised by different phases involving recognition, chemotactic migration, adhesion, ingestion, destruction and elimination of foreign cells (Tiscar and Mosca, 2004). We will focus on properties such as cell adhesion and cell migration through extracellular matrix proteins collagen IV, laminin-1 and on the signaling molecules that mediate these processes.

Cell adhesion, cell migration and extracellular matrix proteins

Among the immune responses in mussels, the processes of hemocyte adhesion to and migration through extracellular matrix play a crucial role in cell immunity. Hemocyte adhesion is an initial step in phagocytosis of foreign particles (Hynes and Lander, 1992). Cell-cell adhesion and cell-substratum adhesion (e.g., to extracellular matrix) are critical for the development, maintenance and

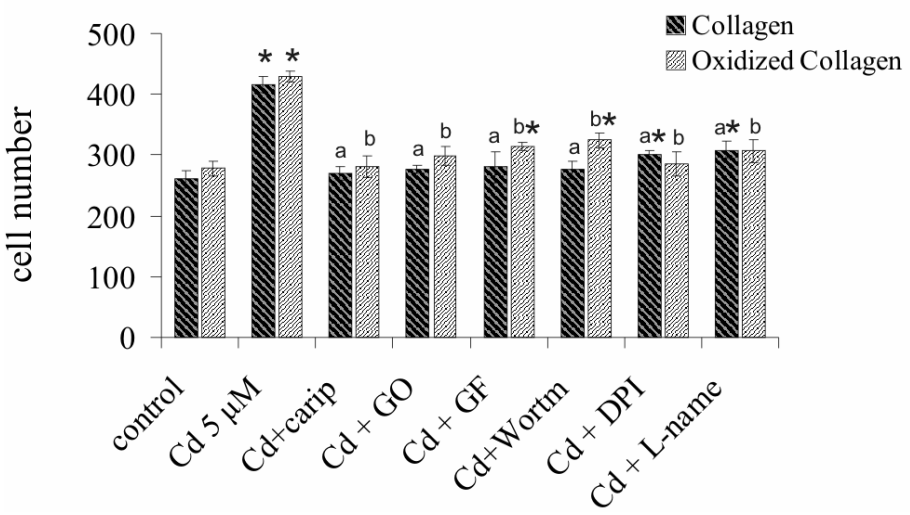

Fig. 2 Migration of *Mytilus galloprovincialis* hemocytes through collagen IV and oxidized collagen IV after treatment with cadmium and inhibitors of NHE, PKC, PI-3K, NADPH oxidase and NO synthase. Hemocytes were pre-incubated with the inhibitors cariporide (20 nM), GO6976 (500 nM) and GF109203X (10 μM) for 15 min at 20 ^{0}C, $CdCl_2$ (5 μM) was then added and the samples were incubated for 30 min at 20 ^{0}C. The results show the means of at least 4 experiments ± SD. The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-hoc test ($p<0.05$). * indicates significant difference of the sample value with the control value. ^{a,b} indicate significant difference between each sample value with the respective control value (Cd alone) (Koutsogiannaki, 2008)

function of multicellular organisms. Moreover, hemocyte adhesion to different surfaces can result in important cellular behaviors such as parasitic encapsulation and hemocyte-mediated clotting responses (Yoshino, 1998). Hemocyte migration depends on directed cytoskeletal reorganization, ion transport membrane recycling by endocytosis and formation of focal adhesion sites with extracellular matrix. *M. galloprovincialis* hemocytes migration through Extracellular matrix (ECM) proteins was reported after heavy metals (Koutsogiannaki, 2008) and interleukin (IL)- 8 affect (Ottaviani, 2000). Chemotaxis was also detected in immunocytes of the hard clam *Mercenaria mercenaria* as a result of bacteria stimuli (Fawcett and Tripp, 1994).

ECM plays a central role in the structure and maintenance of tissue architecture (Adams and Watt, 1993). It is now evident that ECM turnover is a critical step in tissue remodelling that accompanies many physiological as well as pathological processes in vertebrates, invertebrates and plants (Massova *et al*., 1998). The macromolecules that are present in all extracellular matrices include collagen, proteoglycans and glycoproteins (mainly laminins).

The collagens are a family of extracellular matrix proteins involved in cell adhesion, chemotaxis and migration, and the dynamic interplay between cells and collagens regulates tissue remodelling during growth, differentiation, morphogenesis and wound healing. Cells encounter collagen in a number of different ways. Cells may stably adhere to collagen in tissues and thus receive survival signals (e.g., dermal fibroblasts), migrate through the collagen-rich stroma as part of a normal morphogenic process (e.g., mammary gland branching) or in disease (e.g., tumour metastasis), or interact with collagen as a result of injury (e.g., homeostasis). Interestingly, molluscan hemocytes have been reported to be involved in collagen synthesis and extracellular matrix deposition (Serpentini *et al.*, 2000). In accordance with these, studies in sections of integument from bivalve species suggest that molluscan integumental ECM contains collagens similar to type I, IV, V and VI collagens (Corbetta *et al*., 2002). In addition, molluscan motoneurons adhere to laminin and type IV collagen (Wildering et al, 1998). Furthermore, results suggest that hemocytes after treatment with either cadmium or oxidants adhere to and migrate through collagen IV and oxidized collagen IV at a higher degree compared to control cells (Koutsogiannaki, 2008) (Figs 1-4). It is also suggested that *M. galloprovincialis* hemocytes adhere to collagen with the involvement of α2 integrin subunit (Koutsogiannaki, 2008) (Fig. 5)

Apart from collagens, laminins are also components of the extracellular matrix that determine the histoarchitecture and provide cells with biological information. The laminins are the major family of non collagenous heterodimeric glycoproteins that provide an integral part of the structural scaffolding in almost every tissue of an organism. It has been demonstrated that laminins are mainly involved in the organization of the basal membrane network and are also present in cell-associated extracellular matrices. They are involved in multiple physiological processes including cell proliferation, differentiation, migration, adhesion and survival. The laminins are found as trimeric proteins

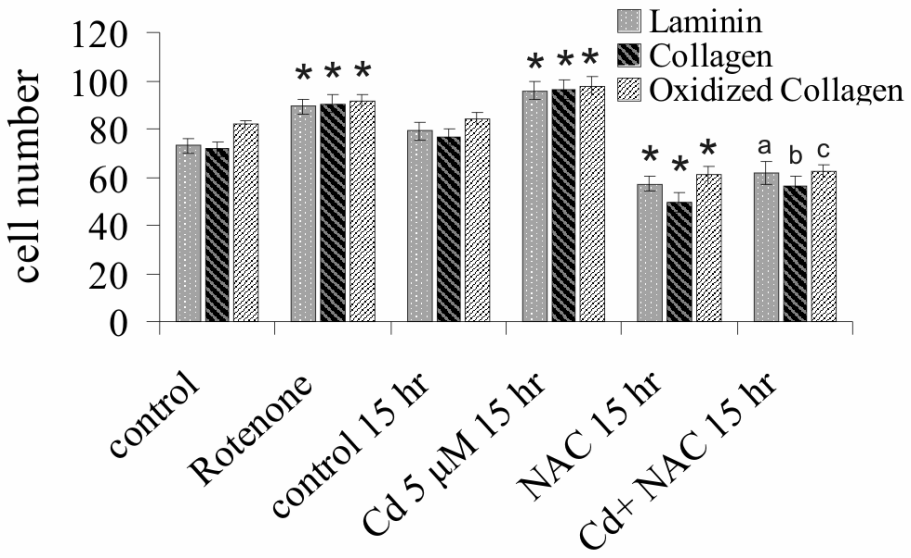

Fig. 3 Adhesion of *Mytilus galloprovincialis* hemocytes to laminin, collagen IV and oxidized collagen IV after treatment with cadmium, oxidants and antioxidants. Hemocytes were incubated with the oxidant Rotenone (25 μM) for 60 min at 20 ^{0}C or with the antioxidant NAC for 15 hr at 20 ^{0}C. The results show the means of at least 4 experiments ± SD. The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-hoc test ($p<0.05$). * indicates significant difference of the sample value with the control value. ^{a,b,c} indicate significant difference between each sample value with the respective control value (Cd alone) (Koutsogiannaki, 2008)

which form a cross, giving a structure that can bind to other cell membrane and extracellular matrix molecules (Timpl and Brown, 1994; Yurchenco and Cheng, 1994; Yurchenco and O'Rear, 1994). It is reported that *M. galloprovincialis* hemocytes after treatment with either cadmium or oxidants adhere to the ECM protein laminin at a higher degree compared to control cells (Koutsogiannaki, 2008) (Figs1, 3).

Hemocyte receptors involved in immune responses

The processes of adhesion and migration are mediated through hemocytes receptors interactions with binding groups. Hemocyte receptors are grouped into several broad groups including lectins (or lectin-like receptors), integrins (or integrin-related receptors) and growth factor/hormone/cytokine-like receptors (Humphries and Yoshimo, 2003).

Lectins, are glycoproteins which serve as recognition molecules by binding to non-self material through carbohydrate recognition sites (Ottaviani, 2006). In addition, a unique family of proteins with CHO-activity, referred to as fibrinogen-related proteins or Freps has been found to be induced in snails in response to infection (Adema *et al*., 1997; Leonard *et al*., 2001). Moreover, selectin-like proteins has been referred to exist in molluscs. Selectins constitute a family of CHO-reactive membrane proteins that are present in endothelial cells, leukocytes and platelets in mammals. They are adhesion receptors involved in many processes such as leucocyte extravascular trafficking and inflammation (Patel *et al*., 2002).

It has also been demonstrated that receptors for platelet-derived growth factor (PDGF-alpha/β) and transforming growth factor β (TGF-β) are present in *M. gallorovincialis* hemocytes involved in many cellular functions such as phagocytosis and cell motility (Ottaviani *et al*., 1997a; Kletsas *et al*., 1998). Moreover, receptors for bioactive peptides such as proopiomelanocortin (POMC) including β-endorphin, adrenocorticotrophic hormone (ACTH) and alpha-melanotropin receptors as well as insulin-like receptors have been found in molluscan hemocytes (Stefano *et al*., 1989; Duvax-Miret *et al*., 1992; Ottaviani *et al*., 1998b; Sassi *et al*., 1998; Lardans *et al*., 2001). Furthermore, cytokine-like receptors have been found to be present in molluscan hemocytes as well. It has been shown that bioactive peptides and cytokines in invertebrates are related to cell shape changes and cell migration (Hughes *et al*., 1990; Ottaviani *et al*., 1995), induce NO synthase (Ottaviani *et al*., 1995) and increase phagocytic activity by activating the classical signal transduction pathways, i.e., protein kinase A, C and B (Ottaviani *et al*., 1997b). Among cytokines, interleukins which belong to chemotactic cytokines also referred as chemokines, are involved in acute inflammation. IL-8 has been demonstrated to induce increased phagocytic activity and chemotactic response in *M. galloprovincialis* hemocytes (Ottaviani *et al*., 2000). Barcia *et al*. (1999) also detected the IL-2 receptor to be present in *M. galloprovincialis* hemocytes.

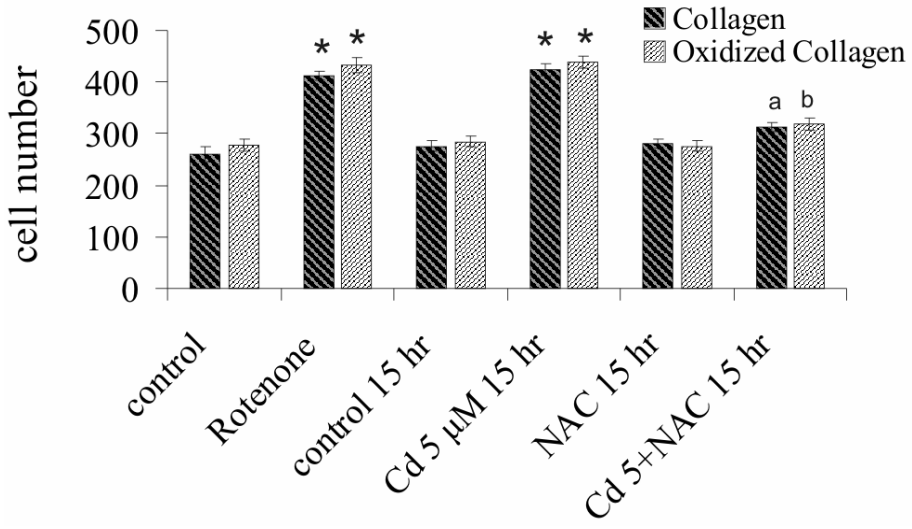

Fig. 4 Migration of *Mytilus galloprovincialis* hemocytes through collagen IV and oxidized collagen IV after treatment with cadmium, oxidants and antioxidants. Hemocytes were incubated with the oxidant Rotenone (25 µM) for 60 min at 20 ^{0}C or with the antioxidant NAC for 15 hr at 20 ^{0}C. The results show the means of at least 4 experiments ± SD. The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-hoc test ($p<0.05$). * indicates significant difference of the sample value with the respective control value. ^{a,b} indicate significant difference between each sample value with the respective control value (Cd alone) (Koutsogiannaki, 2008)

Integrins comprehend a large family of cell surface receptors. In mammals there are integrins binding to laminin ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, α_7,β_1 and $\alpha_6\beta_1$), integrins binding to collagen ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$), integrins of leukocytes ($\alpha L\beta_2$, $\alpha M\beta_2$, $\alpha X\beta 2$ and $\alpha D\beta_2$) and integrins recognizing the RGD motif ($\alpha_5\beta_1$, $\alpha V\beta_1$, $\alpha V\beta_3$, $\alpha V\beta_5$, $\alpha V\beta_6$, $\alpha V\beta_8$ and $\alpha IIb\beta_3$) (Heino *et al*., 2009) Integrins function mainly as cell-matrix adhesion molecules and transducers of the signals between them (Li *et al*., 2003). ECM-integrin interactions function in a bidirectional manner across cell membranes. As the extracellular domain of integrin receptors becomes occupied by ligand and cluster, the integrins set off a cascade of events termed "outside-in" signaling. In this regard integrins orchestrate multiple functions including proliferation, differentiation, gene expression, changes in intracellular pH and death (Ross and Borg, 2001). Moreover, integrins interact with cytoskeleton regulating cell shape and cell migration. These interactions are mediated through binding of the cytoplasmatic domain of integrins to actin network and actin-binding proteins (ezrin, randixin, moesin) (Hynes, 1992). This short cytoplasmatic domain serves also as a host of molecules such as kinases and small GTPases (Ross and Borg, 2001). Integrins are presumed to be present in all the metazoan cells. In invertebrates the structures of integrins are well conserved and functions are correlated with adhesive processes and immune responses (Tanzer, 2006). Studies in molluscan neurons indicate that cells can attach to various substrates using both RGD-dependent and RGD-independent adhesion mechanisms suggesting that at least two different cell adhesion receptors, possibly belonging to the integrin family, are expressed in molluscan neurons (Wildering *et al*., 1998). Results have shown that α_2 integrin subunit mediates the increased adhesion of *M. gallorovincialis* hemocytes to collagen and oxidized collagen induced by cadmium (Koutsogiannaki, 2008) (Fig. 5). In addition increased expression of α2 integrin subunit was observed after cadmium treatment in *M. gallorovincialis* hemocytes, which was due to Na^+/H^+ exchanger (NHE), phosphatidylinositol-3 kinase (PI-3K), protein kinase C (PKC), NADPH oxidase, reactive oxygen species (ROS) and NO involvement (Koutsogiannaki, 2008) (Fig. 6).

Among the adhesion receptors that have been also found in invertebrates are caderins and immunoglobulins (N-CAM) as well as peroxinectin and PSP1 peptide (plasmatocyte spreading peptide) (Johansson, 1999).

Signaling molecules involved in immune responses

The first step to initiate an immune response is the detection by hemocytes of foreign invaders and/or non-self cells, presumably through receptors associated with the surface membrane. Signals generated by ligand binding are then transduced across the membrane resulting in a cascade of downstream chemical reactions, ultimately directing these signals to target organelles (e.g., nucleus, cytoskeleton) mediating the induction of appropriate cellular responses (Heldin and Purton, 1996). Cells

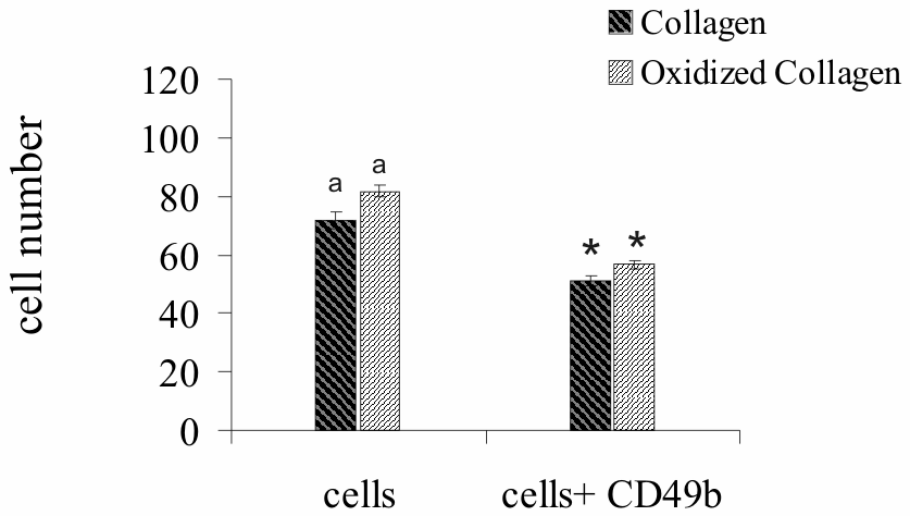

Fig. 5 Adhesion of *Mytilus galloprovincialis* hemocytes to collagen IV and oxidized collagen IV after treatment with the anti-alpha2 integrin subunit. Hemocytes were incubated with the anti-alpha2 integrin subunit (CD49b) for 30 min at 20 ^{0}C. The results show the means of at least 4 experiments ± SD. The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-hoc test ($p<0.05$). * indicates significant difference of the sample value with the respective control value. ^{a} indicates significant difference between each other (Koutsogiannaki, 2008)

mediating immunity are able to communicate with both their internal and external environments through well developed signaling pathways (Hynes and Zhao, 2000). Receptor-ligand interactions result in the modulation of many cellular processes mediated by complex intracellular signal transduction pathways. In invertebrates little is known about these signaling pathways although the cumulative data implies that there is high homology with those of mammals (Humphries and Yoshino, 2003). Studies concerning the induction of the immune system of *M. galloprovincialis* by various stimuli (bacteria, cytokines, hormones, environmental chemicals) suggest the involvement of p38 [(stress-activated p38 mitogen-activated protein kinase (MAPK)], c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription (STAT)- 5, STAT 3, nuclear factor kappa B (NF-kB), PKC, cyclic adenosine monophosphate (cAMP) dependent PKA (cAMP/PKA), PI-3 K, ROS and NO (Ottaviani *et al*., 2000; Canesi *et al*., 2006; Cao *et al*., 2007; Novas *et al*., 2007; Barcia and Ramos-Martinez, 2008; Garcia-Garcia *et al*., 2008; Malagoli *et al*., 2008). In addition, Malagoli *et al*. (2007) reported that stressfull conditions in mussel hemocytes trigger increased phagocytic activity and/or modulation of their signal transduction pathways, mainly ERK and MAP kinases. This flexibility suggests the possibility that accumulated substances exert different effects in diverse situations. On the other hand, Garcia-Garcia *et al*. (2008) suggest that the role of ERK and PKC in phagocytosis regulation is less generalized due to differential stimulation of phagocytic receptors. In addition it has been suggested that different bacteria and bacterial strains can differently affect the host signaling pathways (Zampini *et al*., 2003; Canesi *et al*., 2005, 2006).

Ottaviani *et al*. (2000) suggested that IL-8 triggers conformational changes, induces chemotaxis and increased phagocytic activity in *M. galloprovincialis* hemocytes through PKA and PKC pathway followed by reorganization of the actin microfilaments. This study also suggests that PKA signaling pathway could be more important than the PKC in mediating cell shape changes induced by IL-8. On the other hand, IL-2 mediated biogenin amines (BA) synthesis involves preferably PKC whereas the cAMP dependent PKA plays secondary role (Cao *et al*., 2004, 2007). It has been found that cAMP activates nucleotide depended protein kinases in molluscs (MacDonald and Storey, 1999) and modulates phagocytic behavior of hemocyte (Chen and Bayne, 1995). Results from our laboratory showed that treatment with 3-isobutyl-1-methylxanthin (IBMX), that results in high cAMP cell content, didn't significantly affect the processes of adhesion and migration of *M. galloprovincialis* hemocytes to extracellular matrix proteins laminin and collagen (Koutsogiannaki, 2008). The role of cAMP in these processes warrants further investigation.

NO, different forms of nitric oxide synthase (NOS) and ROS represent some of the main immune mechanisms in invertebrates (Pipe, 1992; Anderson *et al*., 1992; Gourdon *et al*., 2001; Ottaviani, 2006; Barcia and Ramos-Martinez, 2008). ROS are produced through respiratory burst, which is a series of biochemical reactions leading to ROS generation such as superoxide (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) (Cross

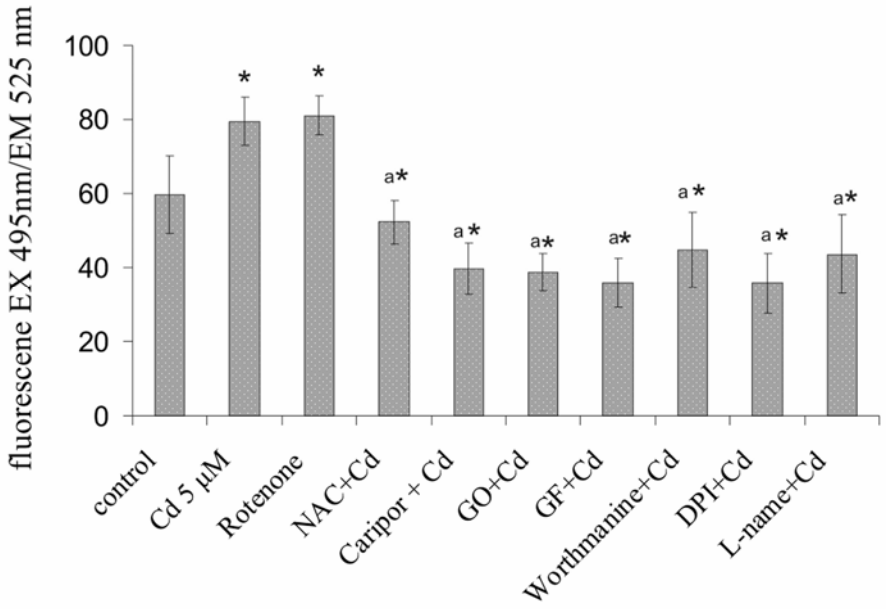

Fig. 6 Integrins expession of *Mytilus galloprovincialis* hemocytes. Hemocytes were incubated with the anti-alpha 2 integrin subunit for 10 min at 20 ^{0}C. The results show the means of at least 4 experiments ± SD. The level of significance of the differences between the samples was calculated by ANOVA with a Student-Newman-Keuls post-hoc test ($p<0.05$). * indicates significant difference of the sample value with the respective control value. [a] indicates significant difference between each sample value with cadmium value (Koutsogiannaki, 2008)

and Segal, 2004). The activation of respiratory burst has been detected in hemocytes of many mollusc species including *M. galloprovincialis* (Garcia-Garcia *et al*., 2008). NO is a highly cytotoxic and microbicidal molecule, that is responsible for the defense mechanisms mediated by macrophages in mammals. It is also capable of activating other leukocytes (Armstrong, 2001). NO synthesis has been demonstrated in many molluscs as well (Ottaviani *et al*., 1993; Arumugam *et al*., 2000; Novas *et al*., 2004; Stefano *et al*., 2004). It has been shown that NO, O_2- and H_2O_2 are involved in the signaling pathway induced by cadmium leading to *M. galloprovincialis* hemocytes adhesion and migration through ECM proteins (Koutsogiannaki, 2008). In addition, the use of oxidants caused increase in adhesion and migration of hemocytes through ECM proteins that was reversed in the presence of the antioxidant NAC (Koutsogiannaki, 2008) (Figs 3, 4). The later observations confirm the fact that ROS are implicated in immune responses of *M. galloprovincialis* hemocytes (Koutsogiannaki, 2008). Furthermore, the use of NOS inhibitors resulted in elimination of the bacteria clumping induced by lipopolysaccharides (LPS) in the molluscan hemocytes of *M. edulis* and *V. alter* (Ottaviani *et al*., 1993). It has been demonstrated that metals can increase ROS production in *M. galloprovincialis* hemocytes with the implication of PKC (Kaloyianni *et al*., 2006). Moreover, in mussel leukocytes NO production seems to be mainly regulated by PI3-K, PKC and ERK families (Garcia-Garcia *et al*., 2008). According to the latter, ERK and PKC regulate NO production only in large semigranular hemocytes as a result of differential membrane phagocytic receptor stimulation. In addition, studies on *Lymnaea stagnalis* relate PKC and ERK to the signaling pathway that regulates NO activity (Wright *et al*., 2006). Moreover, Barcia and Ramos-Martinez (2008) showed that IL-2 induces the synthesis of NO in *M. galloprovincialis* hemocytes via activation mainly of the cAMP dependent PKA and secondary of PKC.

It has been also shown that PI-3K activation plays critical role in the immune responses of *M. galloprovincialis* against pathogens and environmental pollutants (Canesi *et al*., 2002a-c). PI3K has central role in coordinating phagocytosis and is found to mediate production of ROS, NO synthesis and PKC activation in *M. galloprovincialis* hemocytes (Chou *et al*. 1998; Chen *et al*., 2003; Garcia-Garcia *et al*., 2008). In addition, there are studies that point out the role of PI3K in the signaling pathways involved in the interactions of cells with the extracellular matrix in invertebrates and in mammals (Guan and Chen, 1996; Parson, 1996; Wei *et al*., 1997; Howe *et al*., 1998; Koutsogiannaki, 2008; Konstantinidis *et al*., 2009). It has been also reported that treatment with wortmannin (PI3-K inhibitor) caused inhibition of cell adhesion, migration, phagocytosis and reorganization of cytoskeleton in the colonial ascidian *Botryllus schlosseri* (Ballarin *et al*., 2002). Similarly, it has been found that wortmannin effect

caused inhibition of hemocytes adhesion to and migration through ECM proteins (Koutsogiannaki, 2008).

Finally, another signaling molecule that seems to be involved in immune processes is NHE. NHE plays a central role in intracellular pH regulation and homeostasis of cell volume and is also involved in many intracellular signaling pathways (Dailianis and Kaloyianni, 2004; Dailianis *et al*., 2005; Kaloyianni *et al*., 2005; Koutsogiannaki *et al*., 2006). NHE activation is implicated in many other cell functions as cell survival and apoptosis (Koliakos *et al*., 2008). It has been shown that treatment of *M. galloprovincialis* hemocytes with cadmium resulted in increased degree of hemocytes adhesion to and migration through laminin-1, collagen type IV and oxidized collagen type IV in relation to control cells, with the involvement of NHE and PKC (Kaloyianni *et al*., 2006; Koutsogiannaki, 2008). In addition, NHE's implication in cell adhesion and cell migration is probably related to the fact that NHE is involved in focal anchoring sites together with focal adhesion kinase (FAK) and proteins of the actin network (teline, vincoulin, paxiciline and others) through indirect connection with integrins (Beningo *et al*., 2001; Koliakos *et al*., 2001; Webb *et al*., 2002; Stock *et al*., 2005; Kostidou *et al*., 2007) or CD44 (Verfaillie *et al*., 1994).

In conclusion, mussels are able to perform sophisticated responses regarding immune functions. The cumulative data implies the existence of numerous different signaling pathways that may participate in immune responses or the existence of a network of all these suggested pathways, that involve a number of molecules as NHE, PI3-K, PKC, NO, ROS, NADPH oxidase, MAPKs, STATs, JNK, ERK, CREB and NF-kB. Most of the molecules involved in immune processes are well conserved from invertebrates to vertebrates. In the higher forms of life their function remains basically similar. Further research is necessary in order to elucidate the signaling molecules that are involved in these processes and that may lead to a more clear understanding of the immune mechanisms operating in molluscs.

References

Adams JC, Watt FM. Regulation of development and differentiation by the extracellular matrix. Development 117: 1183-1198, 1993.

Adema CM, Hertel LA, Miller RD, Loker ES. A family of fibrinogen-related proteins that precipitate parasite-derived molecules is produced by an invertebrate after infection. Proc. Natl. Acad. Sci. USA 94: 8691-8696, 1997.

Anderson D, Paynter K, Burreson E. Increased reactive oxygen intermediate production by hemocytes withdrawn from *Crassostrea virginica* infected with *Perkinsus marinus*. Biol. Bull. 183: 476-481,1992.

Armstrong R. The physiological role and pharmacological potential of nitric oxide in neutrophil activation. Int. Immunopharmacol. 1: 1501-1512, 2001.

Arumugan M, Romestand B, Torreilles J, Roch P. In vitro production of superoxide and nitric oxide (as nitrite and nitrate) by *Mytilus galloprovincialis* haemocytes upon incubation with PMA or laminarin or during yeast phagocytosis. Eur. J. Cell Biol. 79: 513-519, 2000.

Ballarin L, Scanferla M, Cima F, Sabbatin A. Phagocyte spreading and phagocytosis in the compound ascidian *Botryllis schlosseri*: evidence for an integrin-like, RDG-dependent recognition mechanism. Develop. Comp. Immunol. 26: 345-354, 2002.

Barcia R, Cao A, Arbeteta J, Ramos-Martínez JI. The IL-2 receptor in hemocytes of the sea mussel *Mytilus galloprovincialis* Lmk. IUBMB Life 48: 419-423, 1999.

Barcia R, Ramos-Martinez JI. Effects of interleukin-2 on nitric oxide production in molluscan innate immunity. Inv. Surv. J. 5: 43-49, 2008.

Beningo K, Dembo M, Kaverina I, Small J, Wang Y. Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. J. Cell Biol. 153: 881-887, 2001.

Cajaraville MP, Pal SG, Robledo Y. Light and electron microscopical localization of lysosomal acid hydrolases in bivalve hemocytes by enzyme cytochemistry. Acta Histochem. Cytochem. 28: 409-416, 1995.

Canesi L, Gallo G, Gavioli M, Pruzzo C. Bacteria-hemocyte Interactions and Phagocytosis in Marine Bivalves. Microsc. Res. Tech. 57: 469-476, 2002a.

Canesi L, Betti M, Ciacci C, Scarpato A, Citterio B, Pruzzo C, *et al*. Signalling pathways involved in the physiological response of mussel hemocytes to bacterial challenge: the role of stress-activated p38 MAP kinases. Dev. Comp. Immunol. 26: 325-334, 2002b.

Canesi L, Scarpato A, Betti M, Ciacci C, Pruzzo C, Gallo G. Bacterial killing by *Mytilus* hemocyte monolayers as a model for investigating the signaling pathways involved in mussel immune defence. Mar. Environ. Res. 54: 547-551, 2002c.

Canesi L, Betti M, Ciacci C, Lorusso LC, Gallo G, Pruzzo C. Interactions between *Mytilus* hemocytes and different strains of *Escherichia coli* and *Vibrio cholerae* O1 El Tor: role of kinase-mediated signallling. Cell Microbiol. 7: 667-674, 2005.

Canesi L, Betti M, Ciacci C, Lorusso LC, Pruzzo C, Gallo G. Cell signalling in the immune response of mussel hemocytes. Inv. Surv. J. 3: 40-49, 2006.

Cao A, Ramos-Martı´nez JI, Barcia A. In vitro effects of LPS, IL-2, PDGF and CRF on hemocytes of *Mytilus galloprovincialis* Lmk. Fish Shellfish Immunol. 16: 215-225, 2004.

Cao A, Novas A, Ramos-Martinez JI, Barcia R. Seasonal variations in haemocyte response in the mussel *Mytilus galloprovincialis* Lmk. Aquaculture 263: 310-319, 2007.

Chen JH, Bayne CJ. Hemocyte adhesion in the California mussel (*Mytilus californianus*): Regulation by adenosine. Biochim. Biophys. Acta 1268: 178-184, 1995.

Chen Q, Powell DW, Rane MJ, Singh S, Butt W, Klein JB, *et al*. Akt phosphorylates p47phox

and mediates respiratory burst activity in human neutrophils. J. Immunol. 170: 5302-5308, 2003.
Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen CS, *et al*. Regulation of protein kinase Cζ by PI 3-kinase and PDK-1. Curr. Biol. 8: 1069-1077, 1998.
Corbetta S, Bairati A, Vitellaro Zuccarello L. Immunohistochemical study of subepidermal connective of molluscan integument. Eur. J. Histochem. 46: 259-272, 2002.
Cross AR, Segal AW. The NADPH oxidase of professional phagocytes-prototype of the NOX electron transport chain systems. Biochim. Biophys. Acta 1657: 1-22, 2004.
Dailianis S, Kaloyianni M. Cadmium induces both pyruvate kinase and Na^+/H^+ exchanger activity through protein kinase C mediated signal transduction, in isolated digestive gland cells of *Mytilus galloprovincialis* (L.). J. Exp. Biol. 207: 1665-1674, 2004.
Dailianis S, Piperakis SM, Kaloyianni M. Cadmium effects on ROS production and DNA damage via adrenergic receptors stimulation: Role of Na^+/H^+ exchanger and PKC. Free Radic. Res. 39: 1059-1070, 2005.
Duvaux-Miret O, Stefano GB, Smith EM, Dissous C, Capron A. Immunosuppression in the definitive and intermediate hosts of the human parasite *Schistosoma mansoni* by release of immunoactive neuropeptides. Proc. Natl. Acad. Sci. USA 89: 778-781, 1992.
Fawcett LB, Tripp MR. Chemotaxis of *Mercenaria mercenaria* hemocytes to bacteria in vitro. J. Invertebr. Pathol. 63: 275-284, 1994.
Garcia-Garcia E, Prado-Alvarez M, Novoa B, Figueras A, Rosales C. Immune responses of mussel hemocyte subpopulations are differentially regulated by enzymes of the PI 3-K, PKC, and ERK kinase families. Dev. Comp. Immunol. 32: 637-653, 2008.
Gourdon I, Guerin MC, Torreilles J, Roch P. Nitric Oxide generation by Hemocytes of the Mussel *Mytilus galloprovincialis*. Biol. Chem. 5: 1-6, 2001.
Guan JL, Chen HC. Signal transduction in cell-matrix interactions. Int. Rev. Cytol. 168: 81-121, 1996.
Heino J, Huhtada M, Kapyla J, Johnson MS. Evolution of collagen-based adhesion systems. Inter. J. Biochem. Cell Biol. 41: 341-348, 2009.
Heldin CH, Purton M. Signal transduction. Chapman & Hall, Ltd., London, 1996.
Hine PM. The inter-relations of bivalve hemocytes. Fish Shellfish Immunol. 9: 367-385, 1999.
Howe A, Aplin AE, Alahari SK, Juliano RL. Integrin signalling and cell growth control. Curr. Opin. Cell Biol. 10: 220-231, 1998.
Hughes TKJr, Smith EM, Chin R, Cadet P, Sinisterra J, Leung MK, *et al*. Interaction of immunoreactive monokines (interleukin 1 and tumor necrosis factor) in the bivalve mollusc *Mytilus edulis*. Proc. Natl. Acad. Sci. USA 87: 4426-4429, 1990.
Humphries JE, Yoshino TP. Cellular receptors and signal transduction in molluscan hemocytes: connections with the innate immune system of vertebrates. Integr. Comp. Biol. 43: 305-312, 2003.
Hynes RO. Integrins: Versatility, modulation and signalling in cell adhesion. Cell 69: 11-25, 1992.
Hynes RO, Lander AD. Contact and adhesive specificities in the associations, migrations and targeting of cells and axons. Cell 68: 303-322, 1992.
Hynes RO, Zhao Q. The evolution of cell adhesion. J. Cell Biol. 150: 89-96, 2000.
Johansson MW. Cell adhesion molecules in invertebrate immunity. Dev. Comp. Immunol. 23: 303-315, 1999.
Kaloyianni M, Stamatiou R, Dailianis S. Zinc and 17beta-estradiol induce modifications in Na+/H+ exchanger and pyruvate kinase activity through protein kinase C in isolated mantle/gonad cells of *Mytilus galloprovincialis*. Comp. Biochem. Physiol. 141C: 257-266, 2005.
Kaloyianni M, Ragia V, Tzeranaki I, Dailianis S. The influence of Zn on signaling pathways and attachment of *Mytilus galloprovincialis* hemocytes to extracellular matrix proteins. Comp. Biochem. Physiol. 144C: 93-100, 2006.
Kletsas D, Sassi D, Franchini A, Ottaviani E. PDGF and TGF-beta induce cell shape changes in invertebrate immunocytes via specific cell surface receptors. Eur. J. Cell Biol. 75: 362-366, 1998.
Koliakos G, Trachana V, Gaitatzi M, Dimitriadou A. Phosphorylation of laminin-1 by protein kinase C. Mol. Cells 11: 179-185, 2001.
Koliakos G, Paletas K, Kaloyianni M. NHE-1: a molecular target for signalling and cell matrix interactions. Connect. Tissue Res. 49: 157-161, 2008.
Konstantinidis D, Paletas K, Koliakos G, Kaloyianni M. Signaling components involved in leptin-induced amplification of the atherosclerosis-related properties of human monocytes. J. Vasc. Res. 46: 199-208, 2009.
Kostidou E, Koliakos G, Alamdari D, Paletas K, Tsapas A, Kaloyianni M. Enhanced laminin carbonylation by monocytes in diabetes mellitus. Clin. Bioch. 40: 671-679, 2007.
Koutsogiannaki S, Evangelinos N, Koliakos G, Kaloyianni M. Cytotoxic mechanisms of Zn and Cd involve Na/H exchanger (NHE) activation by ROS. Aquat. Toxicol. 78: 315-324, 2006.
Koutsogiannaki S. Signal transduction pathway induced by cadmium in mussel haemocytes. Postgraduate Diploma Thesis, Aristotle University of Thessaloniki, 2008.
Lardans V, Coppin JF, Vicogne J, Aroca E, Delcroix M, Dissous C. Characterization of an insulin receptor-related receptor in *Biomphalaria glabrata* embryonic cells. Biochem. Biophys. Acta 1510: 321-329, 2001.
Leonard PM, Adema CM, Zhang SM, Loker ES. Structure of two FREP genes that combine IgSF and fibrinogen domains, with comments on diversity of the FREP gene family in the snail *Biomphalaria glabrata*. Gene 269: 155-165, 2001.
Li J, Zhang Y, Kirsner RS. Angiogenesis in wound repair: angiogenic growth factors and the

extracellular matrix. Microsc. Res. Tech. 60: 107-114, 2003.
Little TJ, Hultmark D, Read AF. Invertebrate immunity and the limits of mechanistic immunology. Nat. Immunol. 6: 651-654, 2005.
MacDonald JA, Storey KB. Cyclic AMP-dependent protein kinase: Role in anoxia and freezing tolerance of the marine periwinkle *Littorina littorea*. Mar Biol. 133: 193-203, 1999.
Malagoli D, Casarini L, Sacchi S, Ottaviani E. Stress and immune response in the mussel *Mytilus galloprovincialis*. Fish Shellfish Immunol. 23: 171-177, 2007.
Malagoli D, Casarini L, Ottaviani E. Effects of the marine toxins okadaic acid and palytoxin on mussel phagocytosis. Fish Shellfish Immunol. 24: 180-186, 2008.
Massova I, Kotra LP, Fridman R, Mobashery S. Matrix metalloproteinases: structures, evolution, and diversification. FASEB J. 12: 1075-1095, 1998.
Mix MC. A general model for leukocyte cell renewal in bivalve molluscs. Mar. Fish. Rev. US Natl. Mar. Fish. Serv. 38: 37- 41, 1976.
Mydlarz LD, Jones LE, Harvell CD. Innate immunity, environmental drivers, and disease ecology of marine and freshwater invertebrates. Annu. Rev. Ecol. Evol. S. 37: 251-288, 2006.
Novas A, Cao A, Barcia R, Ramos-Martı´nez JI. Nitric oxide release by hemocytes of the mussel *Mytilus galloprovincialis* Lmk was provoked by interleukin-2 but not by lipopolysaccharide. Int. J. Biochem. Cell Biol. 36: 390-394, 2004.
Novas A, Barcia R, Ramos-Martinez JI. Implication of PKC in the seasonal variation of the immune response of the hemocytes of *Mytilus galloprovincialis* Lmk. and its role in interleukin-2-induced nitric oxide synthesis. IUBMB Life 59: 659-663, 2007.
Ottaviani E, Paemen LR, Cadet P, Stefano GB. Evidence for nitric oxide production and utilization as a bactericidal agent by invertebrate immunocytes. Eur. J. Pharmacol. 248: 319-324, 1993.
Ottaviani E, Franchini A, Cassanelli S, Genedani S. Cytokines and molluscan immune responses. Biol. Cell 85: 87-91, 1995.
Ottaviani E., Franchini A, Kletsas D, Bernardi M, Genedani S. Involvement of PDGF and TGF-beta in cell migration and phagocytosis in invertebrate immunocytes. Anim. Biol. 6: 91-95, 1997a.
Ottaviani E, Franchini A, Franceschi C. Pro-opiomelanocortin-derived peptides, cytokines and nitric oxide in immune responses and stress: an evolutionary approach. Int. Rev. Cytol. 170: 79-141, 1997b.
Ottaviani E, Franchini A, Barbieri D, Kletsas D. Comparative and morphofunctional studies on *Mytilus galloprovincialis* hemocytes: presence of two aging-related hemocyte stages. Ital. J. Zool. 65: 349-354, 1998a.
Ottaviani E, Franchini A, Hanukoglu I. In situ localisation of ACTH receptor-like mRNA in molluscan and human immunocytes. Cell Mol. Life Sci. 54: 139-142, 1998b.
Ottaviani E, Franchini A, Malagoli D, Genedani S. Immunomodulation by recombinant human interleukin-8 and its signal transduction pathways in invertebrate hemocytes. Cell Mol. Life Sci. 57: 506-513, 2000.
Ottaviani E. Molluscan immunorecognition. Inv. Surv. J. 3: 50-63, 2006.
Parson JT. Integrins-mediated signaling: regulation by protein tyrosine kinases and small GTP-binding proteins. Curr. Opin. Cell Biol. 8: 146-150, 1996.
Patel KD, Cuvelier SL, Wiehler S. Selectins: Critical mediators of leukocyte recruitment. Sem. Immunol. 14: 73-81, 2002.
Peteiro LG, Labarta U, Fernandez-Reiriz MJ. Variability in biochemical components of the mussel (*Mytilus galloprovincialis*) cultured after Prestige oil spill. Comp. Biochem. Physiol. 145C: 588-594, 2007.
Pipe RK. Hydrolytic enzymes associated with the granular haemocytes of the marine mussel *Mytilus edulis*. Histochem. J. 22: 595-603, 1990.
Pipe RK. Generation of reactive oxygen metabolites by the haemocytes of the mussel *Mytilus edulis*. Dev. Comp. Immunol. 16: 111-122, 1992.
Renwrantz L. Internal defense system of *Mytilus edulis*. In: Stefano GB (ed), Neurobiology of *Mytilus edulis*, Manchester University Press, Manchester, pp 256-275, 1990.
Rinkevich B, Muller WEG. Invertebrate immunology, Springer, Berlin, 1996.
Ross RS, Borg TK. Integrins and myocrdium. Circ. Res. 88: 1112-1119, 2001.
Sassi D, Kletsas D, Ottaviani E. Interactions of signalling pathways in (1-24)-induced cell shape changes in invertebrate immunocytes. Peptides 19: 1105-1110, 1998.
Serpentini A, Ghayor C, Poncet JM, Hebert V, Galéra P, Pujol JP, *et al*. Collagen study and regulation of the de novo synthesis by IGF-I in hemocytes from the gastropod mollusc, *Haliotis tuberculata*. J. Exp. Zool. 287: 275-284, 2000.
Stefano GB, Leung MK, Zhao X, Scharrer B. Evidence for the involvement of opioid neuropeptides in the adherence and migration of immunocompetent invertebrate hemocytes. Proc. Natl. Aca. Sci. USA 86: 626-630, 1989.
Stefano GB, Kim E, Liu Y, Zhu W, Casares F, Mantiene KJ, *et al*. Nitric oxide modulates microglial activation. Med. Sci. Monit. 10: 17-22, 2004.
Stock CGB, Hauck CR, Arnold H, Mally S, Eble JA, Dieterich P, *et al*. Migration of human melanoma cells depends on extracellular pH and Na^+/H^+ exchange. J. Physiol. 567: 225-238, 2005.
Tanzer ML. Current concepts of extracellular matrix. J. Orthop. Sci. 11: 326-331, 2006.
Timpl R, Brown JC. The laminins. Matrix Biol. 14: 275-281, 1994.
Tiscar PG, Mosca F. Defense mechanisms in farmed marine molluscs. Vet. Res. Commun. 28: 57-62, 2004.
Verfaillie C, Benis A, Iida J, McGlave P, McCarthy J. Adhesion of committed human hematopoietic progenitors to synthetic peptides from the C-

terminal heparin-binding domain of fibronectin: cooperation between the integrin alpha2beta1 and the CD44 adhesion receptor. Blood 84: 1802-1811, 1994.

Webb D, Parsons J, Horwitz A. Adhesion assembly, disassembly and turnover in migrating cell-over and over and over again. Nat. Cell Biol. 4: 97-100, 2002.

Wei J, Shaw LM, Mercurio AM. Integrin sgnaling in leukocytes: lessons from the $\alpha_6\beta_1$ integrin. J. Leukoc. Biol. 61: 397-407, 1997.

Wildering WC, Hermann PM, Bulloch AG. Neurite outgrowth, RGD-dependent, and RGD-indentified molluscan motoneurons on selected substrates. J. Neurobiol. 35: 37-52, 1998.

Wright B, Lacchini AH, Davies AJ, Walker AJ. Regulation of nitric oxide production in *Lymnaea stagnalis* defence cells: a role for protein kinase C and extracellular signal-regulated kinase signalling pathways. Biol. Cell 98: 265-278, 2006.

Yoshino TP. Integrin adhesion receptors in molluscan cells. VIIth International Colloquium on Invertebrate Pathology and Microbial Control and IVth International Conference on *Bacillus thuringiensis*, Sapporo, Japan, pp 277-283, 23-28 August 1998.

Yurchenco PD, Cheng YS. Laminin self-assembly: a three-arm interaction hypothesis for the formation of a network in basement membranes. Contrib. Nephrol. 107: 47-56, 1994.

Yurchenco PD, O'Rear JJ. Basement membrane assembly. Methods Enzymol. 245: 489-518, 1994.

Zampini M, Canesi L, Betti M, Ciacci C, Tarsi R, Gallo G, *et al*. Role for mannose-sensitive hemagglutitin in promoting interactions between *Vibrio cholerae* El Tor and mussel hemolymph. Appl. Environ. Microbiol. 69: 5711-5715, 2003.