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REPORT OF MEETING

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Organizers: **E Ottaviani, D Malagoli**

Department of Animal Biology, University of Modena and Reggio Emilia, Modena, Italy

Session 1. Chairman: E Ottaviani, University of Modena and Reggio Emilia, Modena, Italy

Mussel hemocytes: the role of PKC isozymes in the innate immune response

R Barcia, M Gonzalez-Riopedre, Jl Ramos-Martínez

Department of Biochemistry and Molecular Biology, University of Santiago de Compostela, Campus of Lugo, Lugo, Spain

The ancestral relationship between immune response and stress has evolved from a common cellular space such as the hemocyte of invertebrates to the implication of diverse endocrine elements (HPA axis) in vertebrates.

In marine molluscs, the hemocytes shoot the immune response by means of molecular and physical stimuli that are redundant and pleiotropic. It is well known that different molecules as toxins, cytokines or growth factors induce responses apparently similar, but differing in power, seasonality etc., which proves that every signal has an internal regulating ability.

The subjects of this study were two protein kinases C isolated from *Mytilus galloprovincialis*, namely p60 and p105. Our goal was to investigate their implication in the synthesis of catecholamines, as stress markers, and nitric oxide, associated to the respiratory burst and to phagocytosis, in mussel hemocytes.

Cultured *Mytilus* hemocytes were treated with different agonist and then catecholamine synthesis was monitored. The results obtained were compatible with the modifications that PKCs undergo. IL-2 or LPS activate catecholamine synthesis by the implication of both isoforms of PKC. For its part, PDGF activates dopamine and norepinefrine synthesis through p60, while modifying the balance between p105 active and inactive forms by inhibiting epinefrine synthesis. Also, hemocyte response varied seasonally when activated both by LPS and by IL-2.

Mytilus hemocytes treated with LPS did not show nitric oxide synthesis. On the contrary, IL-2 provoked a remarkable NO production which involved preferably the cAMP-dependent protein kinase (PKA). The use of PKC inhibitors, the study of the seasonal variations, and the detection of an inducible NOS isoform, prove that PKC acts upon the constitutive NOS throughout the year preferably as an inhibitor. In winter, the maximal NO production detected is the result of p105 activating action on a new inducible NOS.

These results prove that the different actions are in accordance with regulating processes through cell signaling mechanisms. On the contrary, they do not support the existence of a specific receptor for each modulator or inducer, thus confirming the hypothesis of the presence of an ancestral common receptor.

Responses of *Mytilus galloprovincialis* hemocytes to heat killed *Vibrio splendidus* LGP32*:* role of p38 MAPK and PKC

C Ciacci[1], B Citterio[2], M Betti[1], B Canonico[1], P Roch[3], L Canesi[4]

[1]*DiSUAN, University of Urbino "Carlo Bo", Urbino, Italy*

[2]*Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Urbino, Italy*

[3] *JRU Ecosystèmes Lagunaires, CNRS-University of Montpellier 2, France*

[4]*Department of Biology, University of Genoa, Genoa, Italy*

Mytilus hemocytes have been recently demonstrated to show differential functional responses to injection of different *Vibrio* species. In particular, in *Mytilus galloprovincialis* hemocytes, differences were observed in responses to *V. splendidus* LGP32, a strain associated with oyster larvae and juvenile stage mortalities, and to *V. anguillarum.*

In this work, the *in vitro* effects of heat-killed *V. splendidus* LGP32 on *Mytilus* hemocytes and the mechanisms involved were investigated. The results were compared with those obtained with *V. anguillarum* (ATCC 19264). Hemocyte adhesion, lysosomal membrane stability, lysozyme release, oxidative burst and NO production were evaluated, as well as the phosphorylation of the stress-

activated p38 MAPK and PKC, that play a key role in the hemocyte response to bacterial challenge. *V. splendidus* did not affect total hemocyte adhesion, but decreased adhesion of large granulocytes, induced lysosomal membrane destabilization, rapid and persistent lysozyme release, stimulation of oxidative burst and NO production. These effects were associated with rapid and persistent activation of p38 MAPK and PKC. In contrast, *V. anguillarum* had no effect on oxidative burst, and induced significantly lower lysozyme release and phosphorylation of p38 MAPK and PKC with respect to *V. splendidus.* These data indicate the existence of specific interactions between *V. splendidus* LGP32 and mussel hemocytes and suggest that this *Vibrio* strain might affect host bivalve cells through disregulation of immune signaling. Overall, the results support the hypothesis that responses of bivalve hemocytes to different bacterial stimuli may also depend on the cell subtype, thus leading to differential activation of different kinases.

Effects of *Vibrio* challenge on digestive gland biomarkers and antioxidant gene expression in *Mytilus galloprovincialis*

C Barmo[1], R Fabbri[1], C Ciacci[2], L. Vergani[1], P Roch[3], G Gallo[1], L Canesi[1]
[1]*Department of Biology, University of Genoa, Genoa, Italy*
[2]*DISUAN, University of Urbino "Carlo Bo", Urbino, Italy*
[3]*JRU Ecosystèmes Lagunaires, CNRS-University of Montpellier 2, France*

In bivalves, functional and molecular responses to bacterial infection have been largely characterized in the immune cells, the hemocytes. Although soft tissues are endowed with bacteriostatic activity, responses at the tissue level, where bacterial infection may cause stressful conditions, have been not specifically investigated.

In this work, the effects of heat-killed *Vibrio* species, *V. splendidus* LGP32 and *V. anguillarum* (ATCC19264), in the digestive gland of *Mytilus galloprovincialis* were investigated. Mussels were injected with either *Vibrio* and tissues sampled at 3, 6 and 24 h post injection (p.i.). Lysosomal biomarkers (Lysosomal membrane stability-LMS and lipofuscin accumulation), activities of antioxidant enzymes (catalase and glutathione transferase-GST) were evaluated, as well as expression of antioxidant molecules (catalase, GST-π and metallothioneins MT10 and MT20) by quantitative RT-PCR were evaluated.

Both *V. splendidus* and *V. anguillarum* significantly affected all parameters measured, to a different extent at different times p.i.. Both *Vibrios* induced similar effects on LMS and antioxidant enzyme activities. In contrast, *V. splendidus* induced a general up-regulation of antioxidant gene expression, whereas *V. anguillarum* did not. The lack of this response was reflected in stronger tissue oxidative stress conditions in mussels challenged with *V. anguillarum*, as indicated by higher lipofuscin accumulation observed at longer times p.i.. The results indicate that *Mytilus* digestive gland can mount an efficient antioxidant response towards *V. splendidus* LGP32, a strain pathogenic to oyster juveniles and larvae. Overall, lysosomal and oxidative stress biomarkers could be usefully applied in order to monitor early changes in the health status of bivalves induced by bacterial infection.

Differential response of *Mytilus galloprovincialis* hemocytes to *in vivo* and *in vitro* bacteria challenge

P Pagliara[1], P Cito[1], P Roch[2]
[1]*Department of Biological and Environmental Sciences and Technology, University of Salento, Lecce, Italy*
[2]*JRU Ecosystèmes Lagunaires, CNRS-University of Montpellier 2, France*

Marine bivalves are filter-feeding organisms able to accumulate a large number of bacteria. The massive presence of bacteria does not affect their survival due to a very efficient immune response. In mussels, hyalinocytes and granulocytes are responsible for cell-mediate immunity. Granulocytes are generally considered to play a prominent role in such defense mechanisms. In this study the response of *Mytilus galloprovincialis* hemocytes to *in vivo* and *in vitro* challenges with two bacteria (*Vibrio anguillarum* and *Micrococcus lysodeikticus*) was evaluated.

Direct injection of live bacteria into the hemolymph induced different hemocyte responses. In particular, the presence of *V. anguillarum* caused morphological changes in hyalinocytes that resulted in apoptosis or in modified cytoplasm containing a large number of vacuoles, while the majority of granulocytes showed a necrotic aspect. Nevertheless some granulocytes resulted rounded with a strongly condensed nucleus. On the other hand, *M. lysodeikticus* induced morphological changes associable to apoptotic process, in both cell types. Also i*n vitro,* bacteria were able to differentially modulate the hemocyte response, being *M. lysodeikticus* able to induce chromatin condensation and rounding of cells and *V. anguillarum* inducing necrosis.

So, the *in vivo* or *in vitro* conditions differently affected the bacteria action, resulting *V. anguillarum* unable *in vitro* to induce apoptotic morphology in hemocytes.

About morphology of apoptotic hemocytes, we evidenced the ability of bacteria to induce a rounding of cells and chromatin condensation, while not relevant nuclear fragmentation, generally considered to be the last step of apoptotic process.

Effects of different environmental *Vibrio* strains on immune responses of *Mytilus* hemocytes

C Ciacci[1], A Manti[1], B Canonico[1], W Baffone[2], R Campana[2], L Canesi[3]
[1]*DiSUAN, University of Urbino "Carlo Bo", Urbino, Italy*
[2]*Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Urbino, Italy*

[3]*Department of Biology, University of Genoa, Genoa, Italy*

Vibrios are Gram (-) autochthonous bacteria of the marine environment, where they occur both free in water and associated with living organism and organic and inorganic surfaces, thus representing a considerable part of heterotrophic bacterial populations. Vibrios are also considered an important cause of human food-borne illnesses associated with the consumption of seafood, including bivalves.

Although in bivalve immunocytes responses to bacterial challenge have been widely investigated, few reports are available on responses of *Mytilus* hemocytes to environmental *Vibrio* isolates.

In this work, the effects of *in vitro* challenge of mussel hemocytes with *V. parahaemolyticus* 80 (isolated from mussel samples of the Conero coast), and *V. alginolyticus* 1513 (isolated from marine plankton), were compared with those of available reference strains, *V. parahaemolyticus* ATCC 43996 and *V. vulnificus* 509. The results indicate differential responses to different vibrios in terms of lysosomal membrane destabilization and activation of immune parameters (lysozyme release, oxidative burst and NO production). *V. parahaemolyticus* strains, and in particular the *V. parahaemolyticus* 80 isolate, producing the virulence factor TDH (thermostable direct hemolysin), induced stronger lysosomal destabilization and smaller activation of immune responses in comparison to other vibrios. Flow cytometry analysis showed that different vibrios did not affect total hemocyte counts (THC), but induced changes in hemocyte subpopulations.

These results support the hypothesis that *Mytilus h*emocytes represent a sensitive target for the action of different *Vibrio* species and strains and indicate that these invertebrate cells can be utilized for elucidating the mechanisms for pathogen action also in vertebrates.

Changes in hemocyte subpopulations and immunoreactivity to anti-integrins and -progenitors antibodies of *Mytilus galloprovincialis* after bacterial challenge

B Canonico[1], C Ciacci[1] , M Arcangeletti[1], M Betti[1], B Citterio[2], P Roch[3], S Papa[1], L Canesi[4]
[1]*DiSUAN, University of Urbino "Carlo Bo", Urbino, Italy*
[2]*Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Urbino, Italy*
[3]*JRU Ecosystèmes Lagunaires, CNRS-University of Montpellier 2, France*
[4]*Department of Biology, University of Genoa, Genoa, Italy*

The involvement of circulating hemocytes as the principal cellular effector mediating molluscan immune responses is well established. Microbial invasion poses an immediate threat to survival, and a vigorous defense response ensues an effort to clear the pathogen from the host. Overall, experimental evidence suggests that molluscan immune responses rely on molecules that share homology with those of vertebrate systems. In this work, flow cytometric techniques and anti-human mouse monoclonal antibodies (mAbs) were utilized to monitor changes in hemocyte subpopulations of *Mytilus galloprovincialis*, after bacterial challenge, *in vivo* and *in vitro*. We focused on total hemocyte count (THC), FSC and SSC physical characteristics, positivity to anti CD34, anti CD117 and anti CD11b antibodies. These reagents in humans recognized stem and progenitor cells (CD117 and CD34) and neutrophils/monocytes (CD11b). We applied cytometric internal controls to monitor cell autofluorescence, avoid artifacts due to formaldehyde fixation. The results show that 6h after bacterial challenge, a decrease in large granulocyte subpopulation occurs, particularly with *Vibrio splendidus*. Furthermore, we found a different expression of the antigens investigated in hyalinocytes, small and large granulocytes. Inoculation of *V. splendidus*, *V. anguillarum* and *Micrococcus lysodeitkicus* seems to produce a rearrangement in the distribution of these antigens. These *in vivo* data are also substantiated by the adhesion test after *in vitro* bacterial challenge. This study confirm the existence of heterogeneity among circulating hemocytes, the presence of differentially engaged cell populations during bacterial challenge and a kind of proliferation/differentiation pathway differently primed by various bacteria.

Functional differential hemocytes behaviour in the clearance of bacteria and humoral defense factors variability in *Mytilus galloprovincialis*

MG Parisi[1], M Cammarata[1], H Li[2], M Toubiana[2], N Parrinello[1], P Roch[2]
[1]*Department of Animal Biology, University of Palermo, Palermo, Italy*
[2]*JRU Ecosystèmes Lagunaires, CNRS-University of Montpellier 2, France*

Bivalve molluscs are constantly exposed to various pathogen agents. To survive in the aquatic environment, they have developed active cellular and humoral immune responses.

In this study, flow cytometry was used to identify three hemocytes sub-populations in *M. galloprovincialis*: hyalinocytes, small and large granulocytes. When bacteria of *Vibrio* and *Micrococcus* genus were injected into mussel circulation, proportions of the three cells categories varied differentially and increment of living intra-hemocyte bacteria number suggested intense phagocytosis. Lysozyme gene expression study during bacterial infection and in vivo heat shock and cold temperature treatment showed that hemocyte populations were also capable of discriminating between stress factors and pathogenic species. Finally sequence diversity in antimicrobial peptides (AMP) from Mediterranean mussels were found. In addition, Mytilin B mRNA appeared to be translated into propeptide and his mature form obtained inside hemocyte granules. Polymorphism observed at specific locations indicated negative selection pressure on signal and mature peptide domains. However, a positive selection pressure for COOH-terminus domain can be suggested. Studies are in progress to explain the relationship between

different environment conditions and mussel AMPs polymorphism.

Variability of antimicrobial peptides in *Mytilus galloprovincialis*

U Rosani[1], L Varotto[1], A Rossi[1], B Novoa[2], P Roch[3], A Figueras[2], P Venier[1]
[1]Department of Biology, University of Padua, Padua, Italy.
[2]Instituto de Investigaciones Marinas (CSIC), Vigo, Spain
[3]JRU Ecosystèmes Lagunaires, CNRS-University of Montpellier 2, France

In *Mytilus spp.* four different antimicrobial peptide (AMP) families have been described. AMPs are produced both constitutively and in response to vaious stimuli, and show complex expression patterns in the mussel hemocytes. In fact, AMPs precursors can represent 25-40 % of the whole hemocyte transcriptome and a remarkable sequence variability, with unique profiles in individual mussels, has been reported for myticin C.

Aiming to investigate the natural variability of AMP transcripts in farmed mussels we adopted a high throughput sequencing approach which allows the detection of rare sequence variants. Primers were carefully designed to cover almost all the known sequence variants (cds) of mytilin, myticin, defensin and mytimycin. Following PCR amplification, the tagged amplification products were sequenced with a Genome Sequencer FLX™ system.

At first glance, the variability of the AMP transcript precursors is comparable to that reported for the Myticin C and mainly represented by single nucleotide substitutions (sequence variability of the mature peptides is substantially reduced). Specific patterns of variation will be compared with appropriate statistical tests and the overall analysis will also include the comparison between different geographical regions.

Session 2. Chairman: L Ballarin, University of Padua, Padua, Italy

Cytokines do exist in invertebrates!...Now what?

D Malagoli
Department of Animal Biology, University of Modena and Reggio Emilia, Modena, Italy

Cytokines are soluble mediators of a relatively small molecular weight mainly produced by the cells of the immune or neuroendocrine systems during immune response. Cytokines have been described principally in mammals, even though during 80's and 90's several authors have reported the presence of cytokine-like molecules in invertebrates. The existence of cytokines in protostomians was only confirmed in the last decade, when molecular biology and functional studies lead to the discovery of Spätzle and Unpaired (Upd)-3 in *D. melanogaster*, Hemocyte Chemotactic Peptide (HCP) in the moth *Pseudaletia separata* and Astakine 1 in the freshwater crayfish *Pacifastacus leniusculus*. These findings have finally demonstrated that cytokines exist in invertebrates and probably several other factors will be discovered in the next future. However, the fundamental questions pertaining the evolution of cytokines (i.e., which is their origin and how their differentiation have proceeded) remain unanswered.

The analysis of cytokine genes in mammals have demonstrated the extreme variability of the cytokine sequences, especially of interleukins. This makes the study of cytokine evolution a very difficult task, that requires specific algorithms to find potentially conserved molecules and full molecular and structural characterization as a final step. In this perspective, molecules such as *Drosophila* Helical Factor (DHF) may prove of help in describing the evolution of one of the major classes of immune-related molecules, but further studies are required before we gain the knowledge necessary to trace a draft of a phylogeny tree for cytokines.

Implication of an IL-16 homologous in microglial cells recruitment after CNS injury in the medicinal leech *Hirudo medicinalis*

J Vizioli, F Croq, A Garçon-Bocquet, M Tahtouh, P-E Sautière, C Van Camp, M Salzet, J Pestel, C Lefebvre
Laboratory of Annelids Neuroimmunology, CNRS FRE 2933, University of Lille1, Cité Scientifique Bat. SN3 59655 Villeneuve d'ascq, France

The medicinal leech *Hirudo medicinalis* can totally repair its central nervous system (CNS) after injury. This invertebrate model offers unique opportunities to study the molecular and cellular basis of the CNS repair processes. When the leech CNS is injured, microglial cells migrate and accumulate at the site of lesion, this phenomenon is essential for the usual sprouting of injured axons. We recently isolated a novel molecule, named *Hm*IL-16, having homologies with human interleukin-16 (IL-16) active form. *Hm*IL-16 has a chemotactic activity on leech microglial cells similar to that observed using a gradient of human IL-16.

The pre-incubation of microglial cells either with an anti-human IL-16 or with anti-*Hm*IL-16 antibodies highly reduced the leech conditioned medium-mediated microglia migration. Moreover, *Hm*IL-16 was demonstrated to promote human $CD4^+$ T cells migration. Using either antibody against human IL-16, an IL-16 antagonist peptide or soluble CD4, human $CD4^+$ T cells migration promoted by *Hm*-IL-16 was reduced. In leech, immunohistochemistry studies on CNS suggests that *Hm*IL-16 protein present in the neurons is rapidly transported and stored along the axonal processes to promote the recruitment of microglial cells close to injured axons. To our knowledge, this is the first time that an interleukin-16 has been found in CNS invertebrate with an associated biological function.

A homologous of Interleukine-16 is involved in leech wound healing

A Grimaldi[1], C Bianchi[1], G Greco[1], G Tettamanti[1], R Valvassori[1], M de Eguileor[1], A Garçon-Bocquet[2], C Lefebvre[2], J Vizioli[2]

[1]*Department of Biotechnology and Molecular Sciences, University of Insubria, Varese, Italy*
[2]*Laboratory of Annelids Neuroimmunology, CNRS FRE 2933, University of Lille1, Cité Scientifique Bat. SN3 59655 Villeneuve d'ascq, France*

Several reports in these recent years have highlighted that cytokines and their signalling pathways have been highly preserved during evolution. We have already described the remarkable conservation of these molecules and their related function in leeches as well. Recently a new cytokine has been identified in the leech *Hirudo medicinalis*. This molecule is homolog to human interleukin-16 (IL-16) an it is involved in the central nervous system repair. Since in vertebrates IL-16 plays an important role in innate immune responses and is a major chemotactic signal for $CD4^+$ cells, we focused our study on the possible role of *Hm*IL-16 in the regulation of inflammation and wound healing processes in leech as well. In particular we investigated on the expression of *Hm*IL-16 in the two tissue of leech mainly involved in these processes: the botryoidal tissue, involved in the myelo/erythroid and hematopoietic cell production and in the angiogenic activity, and the vasofibrous tissues, responsible in the formation of the pseudoblastema during wound healing processes.

We found that *Hm*IL-6 injection in the leech body wall induces the proliferation and migration, towards the stimulated area, of the vasofibrous tissue cells. This type of cells highly express both *Hm*IL-16 and its receptor $CD4^+$. The chemoattractant activity of *Hm*IL-16 for leech immune cells $CD4^+$ was validated by using the innovative technique of matrigel biopolymer supplemented with *Hm*IL16 and injected in leech body wall.

Immune response of *Anopheles stephensi* immunocytes to *Asaia* infection

M Mandrioli
Department of Animal Biology, University of Modena and Reggio Emilia, Modena, Italy

The acetic acid bacteria belonging to the genus *Asaia* have been recently identified in midgut, salivary glands and reproductive organs of the Asian malaria vector *Anopheles stephensi*. *Asaia* has been proven to be easily cultivable and transformable, and modified strains of *Asaia* expressing green fluorescent protein (GFP) or red fluorescent protein (DsRed), efficiently colonize recipient mosquitoes. *Asaia* is horizontally transmitted to members of mosquito populations by co-feeding and mating and by maternal and paternal vertical transmission routes. Even if the transmission routes are quite well established, at present it is not clear how *Asaia* can move from the gut to the salivary glands and the reproductive organs without being killed by the host immune system. At this aim, immunocytes have been isolated from *A. stephensi* adult mosquitoes and maintained in culture in order to test their response to *Asaia*. In particular the effect of *Asaia* on the transcriptional levels of cecropin, defensin and gambicin genes has been evaluated by RT-PCR. Moreover, phagocytosis tests have been performed in order to verify if *Asaia* could activate this process. On the basis of the results, the symbiont *Asaia* induces an immune response that is similar to that observed after *Escherichia coli* induction, but *Asaia* is not phagocytized at the contrary of what happens with *E. coli*. It could be therefore very intriguing to verify if *Asaia* is killed by the antimicrobial peptides whose transcription is increased in response to *Asaia* infection or if *Asaia* is resistant to these peptides.

Proliferation and differentiation of mussel hemocytes are conserved from molluscs to mammals

M Betti[1], C Ciacci[1], P Gobbi[1], B Canonico[1], S Papa[1], L Canesi[2]
[1]*DISUAN, University of Urbino "Carlo Bo", Urbino*
[2]*Department of Biology, University of Genoa, Italy*

Stem Cell Factor (SCF) is a cytokine that binds to the c-Kit receptor (CD117). SCF can exist both as a transmembrane protein and a soluble protein. This cytokine plays an important role in hematopoiesis, spermatogenesis, and melanogenesis. Soluble and transmembrane SCF bind to c-Kit and are biologically active. Both forms of SCF is produced by fibroblasts and endothelial cells. Soluble SCF has a molecular weight is 18.5 kDa and forms a dimer. SCF plays an important role in the hematopoiesis during embryonic development. SCF plays a role in the regulation of hematopoietic stem cells (HSCs): SCF has been shown to increase the survival of HSCs *in vitro* and contributes to the self renewal and maintenance of HSCs *in vivo*. SCF binds to the c-Kit receptor (CD 117), a receptor tyrosine kinase. c-Kit is expressed in HSCs, mast cells, melanocytes, and germ cells. It is also expressed in hematopoietic progenitor cells including erythroblasts, myeloblasts, and megakaryocytes. SCF binding to c-Kit causes the receptor to homodimerize and auto-phosphorylate at tyrosine residues. The activation of c-Kit leads to the activation of multiple signaling cascades, including the RAS/ERK, PI3-Kinase, Src kinase, and JAK/STAT pathways. Flow cytometry analysis of control hemocytes of *Mytilus sp*. showed immunoreactivity towards CD34 and CD90 antibody utilised has a markers of HSCs. SCF-treated mussel hemocytes showed increase in phagocytic activity, reduction in lysosomal membrane fusion processes and increase expression of cyclin A and D. Ultrastructural morphological studies in colcemide-treated hemocytes confirmed that the mussel cells are able to enter the mitotic phase of the cell cycle. Confocal microscopy analysis of control and SCF-treated hemocytes showed that these effects of SCF involved the activation of c-Kit tyrosine kinase-like receptors.

New insight into the genetic basis of the high-multiple mating type systems of the modern species of the ciliate *Euplotes*

A Vallesi[1], C Alimenti[1], S Federici[2], G Di Giuseppe[2], F Dini[2], P Luporini[1]

[1]*Department of Enviromental and Natural Sciences, University of Camerino, Camerino, Italy*
[2]*Department of Biology, University of Pisa, Pisa, Italy*

The high-multiple ("open") systems of mating types (MT) that control self/non-self recognition in the most recently evolved complex of *Euplotes* species are traditionally considered to be genetically determined by series of single-locus alleles designated as *mat*-1, *mat*-2, *mat*-3, and so forth. These genes are inherited and expressed accordingly to a Mendelian mechanism of serial dominance (i.e., *mat*-1 > *mat*-2 > *mat*-3 and so forth). Therefore, the heterozygous condition (e. g., *mat*-1/*mat*-2) would mimic the corresponding dominant condition (i. e., *mat*-1/*mat*-1), both gene combinations expressing the same phenotype MT-I due to the production of only one chemical signal (pheromone) specified by the dominant gene *mat*-1. Working on a paradigmatic modern species, *E. crassus*, we first characterized the amino acid sequence of a pheromone (designated as *Ec*-ph1) purified from the culture filtrates of the strain L2D. Based on the knowledge of this sequence, we synthesized oligos for PCR-cloning the *Ec*-ph1 gene from the strain L2D, as well as other pheromone genes from other *E. crassus* strains. We obtained evidence that the *Ec*-ph1 gene: (i) coexists in the strain L2D with a second pheromone gene (*Ec*-ph2) which is structurally divergent from the *Ec*-ph1 gene and equally expressed; (ii) is present and co-expressed also in other *E. crassus* strains in combination with other homologous (allelic) pheromone genes (*Ec*-ph3, *Ec*-ph4, and so forth). These observations thus imply that in the modern species of *Euplotes,* as is the case in the ancient ones, the multiple series of mat genes are regulated by relationships of co-dominance rather than of serial dominance. Crucial insights to understand the functional differences between the two models await a definitive characterization of the expression and activity of the *Ec*-ph1 pheromone gene in multiple *E. crassus* strains.

An unusual chordate metallothionein gene in *Ciona intestinalis* genome: structure and expression studies

N Franchi, M Del Favero, E Piccinni, L Ballarin
Department of Biology, University of Padua, Padua, Italy

Metallothioneins (MTs) are able to bind essential and non-essential heavy metal ions, thus controlling cellular homeostasis and detossification. In addition, they act as scavengers for reactive oxygen species (ROS), thanks to their abundant thiols groups. They have a role also in the regulation of inflammatory responses through the modulation of immunomodulatory humoral components.

Chordata represents the major phylum of Deuterostomes, including about 45,000 species distributed in three subphyla: Tunicata (Urochordata), Cephalochordata and Vertebrata. Invertebrate Chordata, about 3 % of the total chordate species, are collectively named Protochordata. Unfortunately, no MT genes have been annotated so far in Protochordates. In order to allow a comparison with the vertebrate MTs we undertook a search for MT genes in the genome of the solitary tunicate *Ciona intestinalis*. We were able to find a MT gene (CiMT1), which represents the first MT gene identified in Tunicates. Its expression is limited to hemocytes and modulated by Cd, Zn and Cu. The deduced protein is only 39 amino acids in length with no typical α and β domains. However the sequence shows that this protein shares the usual percentage (≥ 30) of Cys residues arranged in typical conserved motifs reported for vertebrates.

Further insight on *Ciona intestinalis* prophenoloxidase system activated during the LPS induced inflammatory response

M Cammarata, V Mangano, MR Trapani, V Arizza, A Vizzini, D Parrinello, M Vazzana, M Pergolizzi, N Parrinello
Department of Animal Biology, University of Palermo, Palermo, Italy

In invertebrates, activated phenoloxidases participates in the melanization and is involved in different biological activities including the immune responses. A contact with foreign agents activates, through a serine protease pathway, challenges the prophenoloxidase system (proPO) producing the active form of the enzyme (PO). In *Ciona intestinalis*, the PO is a o-diphenoloxidase contained in hemocytes. In the present work we report on the proPO system and related molecules in *C. intestinalis* tunic during the LPS induced inflammatory responses with particular attention to the biochemical, cellular and molecular aspects. Following an inflammatory stimulus numerous hemocytes infiltrate the inflamed tissue, degranulate contributing to inflammatory events and capsule formation. The tunic inflammatory cells containing phenoloxidases were identified by microscopic observations, tunic explants were assayed for the enzymatic reaction, and immunohistochemistry performed with specific antibodies. HPLC purification of PO extracted from the tunic supported the high molecular weight of native phenoloxidase as well as the enhancement in its activity following treatment with trypsin. For the first time a stable PO form was purified from ascidians allowing further functional studies. Analysis of separated fractions showed that the PO activity after LPS injections is due to modulation of two components different in size. The possibility exist that, as already reported in other invertebrates, the enzyme activation event was due to multiple subunits molecular association.

Finally, the genes for the PO1, and PO2, peroxinectin and Cu-Zn superoxide dismutase, were identified *in silico,* the presence in hemocytes demonstrated by PCR mRNA amplification, and the reaction product sequenced.

Why animals invented endogenous morphine before plants?

D Sonetti[1], B Okere[1], E Bianchi[2]
[1]*Department of Animal Biology, University of Modena and Reggio Emilia, Modena, Italy*

[2]*Department of Neurosciences, Section of Pharmacology, University of Siena, Siena, Italy*

Morphine, the most experimented alkaloid in human history, is commonly thought to derive from poppy plant, *Papaver somniferum*. Its biosynthetic pathway includes several enzymatic steps starting from tyrosine and passing through dopamine as intermediate. The presence and origin of endogenous morphine even in animal tissues is now well documented. Its role as communication molecule has been mainly demonstrated in nervous and immune systems both in vertebrates and invertebrates where a similar biosynthetic pathway has been postulated. In previous studies, an evolutionary linearity was suggested indicating the origin of morphine in plants than, following the same pathway, in invertebrates in which the origin of cathecolamines might start from the intermediate dopamine with production of nor-epinephrine and finally in the vertebrates where the final step is the production of epinephrine. Recently, some data indicate that morphinergic neurons should start the pathway directly from dopamine even if they do not produce catecholamines by themselves. We now hypothesize that morphinergic neurons import from extracellular space dopamine to produce morphine and preliminary data indicate the possible presence of dopamine transporters on morphinergic neurons. In the light of our results we now question the previous evolutionary hypothesis even because a look to the appearance of Angiospermae (to which belongs the genus *Papaver*) put them in a geological period in which not only the most of invertebrates were already present but also evolved vertebrate like Reptiles were too. Endogenous morphine, thus, was "invented" by animals as important signaling molecule and the discovering of this narcotic in plants by man and its use (and abuse) was just a secondary fact.

Session 3. Chairman: N Parrinello, University of Palermo, Palermo, Italy

Invertebrate immunity: what remains to be learned about the detection and destruction of non-self

AJ Nappi
Department of Biology, Loyola University Chicago, Chicago, Il 60525, USA

Invertebrate host-parasite interactions have been the subject of numerous investigations involving physiological, biochemical, molecular, ecological, and behavioral components. The dynamic and varied competing interactions of several species of *Drosophila* and their endoparasitic wasps have been helpful in our attempts to analyze and compare whole genome microarray data with transcriptional responses. Genetic manipulations of virulent and avirulent parasitoids with both resistant and susceptible hosts have provided limited insight into the nature of the killing molecules, some understanding of the immune signaling pathways employed, and possibly some likely targets of immune suppression by invading pathogens. Our inability to directly link altered gene expression with translational events that are indisputably involved in pathogen destruction or immune suppression represents an enduring challenge for future studies.

Cellular and biochemical responses in experimentally-stressed crabs (*Carcinus aestuarii*): effects of bacterial challenge, leg ablation and starvation

V Matozzo, C Gallo, MG Marin
Department of Biology, University of Padua, Padua, Italy

In the first experiment, crabs (*Carcinus aestuarii*) were challenged *in vivo* with *Micrococcus lysodeikticus*, and hemolymph was collected after 24 h. In the second experiment, crabs were subjected to leg ablation, and hemolymph was collected after 1, 3 and 7 days. In the third experiment, crabs did not receive food for 7 days, and hemolymph was then collected. Total hemocyte count (THC), cell proliferation, phenoloxidase (PO) activity in both hemocyte lysate (HL) and cell-free hemolymph (CFH), and CFH glucose levels were evaluated. In all the experiments, the above responses were measured at the same time in both treated and control crabs and then compared.

No significant variation in THC was observed between bacteria-injected crabs and controls, whereas cell proliferation and glucose levels increased significantly in the former when compared to the latter. No significant variation in PO activity was observed between bacteria-injected crabs and controls. One-day after leg ablation, significantly increased THC was found in de-clawed crabs. In de-clawed crabs, cell proliferation was significantly higher after 7 days, whereas glucose and CFH PO activity significantly increased after 1 and 3 days. Starvation caused a statistically significant increase in THC after 7 days, whereas no significant differences in cell proliferation were observed in starved and control crabs. Glucose levels in hemolymph and PO activity in HL significantly increased in starved organisms with respect to controls. Overall, results obtained demonstrated that stress conditions induced in *C. aestuarii* can alter both cellular and biochemical responses investigated, suggesting differing immunomodulation pathways depending on the type of stress undergone.

Crayfish hemocyte type classification via SR infrared microspectroscopy

S Lorenzon[1], PG Giulianini[2], A Mosco[2], L Vaccari[3]
[1]*Department of Biological Oceanography, National Institute of Oceanography and Experimental Geophysics, Trieste, Italy*
[2]*Department of Life Science, University of Trieste, Trieste, Italy*
[3]*ELETTRA Synchrotron Light Laboratory, Area Science Park, Trieste, Italy*

The circulating hemocytes of crustaceans play a central role in innate immunity. Three hemocyte types are commonly described in crustaceans: hyaline hemocytes (HH), small granule hemocytes (SGH), and large granule hemocytes (LGH). Recently, we have identified in the freshwater crayfish *Astacus leptodactylus* a fourth type, medium diameter granule hemocytes (MGH), that represents about 4 % of total circulating hemocytes. HH are involved in hemolymph clotting, while the granular cells contribute to the humoral immune response through the release of immune factors and phagocytosis.

The traditional morphological crustacean hemocyte classification is based essentially on thresholds of granule number and size and on nucleus to cell ratio using phase contrast or brightfield light microscopy and transmission electron microscopy.

In this work we used an original approach to crustacean hemocyte classification based on Synchrotron Radiation (SR) InfraRed MicroSpectroscopy (IRMS). IRMS is an absorption spectroscopy, well established as a sensitive bio-analytical tool for the characterization of the chemical composition of the tested samples. The analysis gives new information on the spatial distribution of different cellular macromolecule constituents (proteins, lipids, nucleic acids and carbohydrates), holding also to a possible clarification of the origin (single or different cell lines) of the circulating hemocytes.

A response of *Rhynchophorous ferrugineus* (Coleoptera: Curculionidae) larval hemocytes to *Bacillus thuringiensis*

B Manachini, V Arizza, D Parrinello, N Parrinello
Department of Animal Biology, University of Palermo, Palermo, Italy

Rhynchophorous ferrugineus Olivier larvae are a pest of palm trees who are still difficult to combat with both chemical and biological control. The bacterium *Bacillus thuringiensis* (Bt) is a pathogen of many insect species and is actively used in biocontrol. Little is known on the interaction of pathogens with the defense responses of these insects. Insect circulating hemocytes are primarily responsible for the immune defense against parasites and pathogens. Here, we report on the response of *R. ferrugineus* (5th-instar nymphs) circulating hemocytes following Bt spore ingestion and vegetative form inoculation. In the hemolymph, plasmatocytes, granulocytes, prohemocytes, oenocytes and spherulocytes were identified. After ingestion of a sub lethal dose (LC_{50}), of commercial product based on spores of Bt, RPW larvae after Bt treatment lose 30 % in weight and had a decrement in the total number of circulating hemocytes. Particularly there was a reduction in the plasmatocytes which also lost their typical spindle-shape. Many specimens of vegetative forms of Bt were found in RPW larvae fed with Bt spores. The vegetative form as been reported as involved in insect septicemia process. The decrease in the hemocyte number was also found following Bt (vegetative form)-inoculation. The percent plasmatocytes was drastically reduced. However further research are necessary to clarify the role of plasmatocytes in the larvae and their interaction with Bt.

Anti biofilm activity of *Paracentrotus lividus* coelomocytes

V Arizza[1], D Schillaci[2], S Molin[3], N Parrinello[1]
[1]Department of Animal Biology, University of Palermo, Palermo, Italy
[2]Department of Medicinal Chemistry and Technology, University of Palermo, Palermo, Italy
[3]Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark

Defense system of marine invertebrates is based solely on the innate immune system that includes both humoral and cellular responses. Antimicrobial peptides (AMPs) are a major component of the humoral immunity, and they display broad antimicrobial activities with remarkable specificity for prokaryotes. AMPs have been found to exert an antimicrobial activity also against human pathogens. They are characterized by a small size due to 10-50 amino acids provided with positive or amphipathic charges. In this work, we isolated a peptide fraction from the coelomocyte supernatant of sea-urchins *Paracentrotus lividus* (5-CC) showing a mass not exceeding 5 kDa. This fraction displayed *in vitro* a wide spectrum of antimicrobial activity against all strains of human pathogens bacteria tested, such as *Staphylococcus aureus* ATCC 29213, ATCC 25923, ATCC 43866, *Staphylococcus epidermidis* DSM3269, 1457, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231 and *Candida tropicalis* ATCC 13813. The minimum inhibitory concentration (MIC) of 5-CC varies from 253.7 to 15.8 mg/ml^{-1}. In addition, as shown through a confocal microscope, the 5-CC also inhibited *Staphylococcus* biofilm. To characterize the AMP contained in the peptide fraction, the 5-CC was analysed with a RP-HPLC/nESI-MSMS. Three main peptides (Parcentrin I, II and III) disclosing 1251.7, 2088.1 and 2292.2 daltons molecular size were identified. The MSMS analysis showed that these peptides include the aminoacid sequences 9-19, 12-31 and 24-41 of ß-thymosin from *P. lividus* (AN AJ439718) respectively. ß-thymosin is an antibacterial peptide contained in vertebrates platelets. Research in progress will disclose the AMP expression by hemocytes and tissues, moreover *in vivo* and *in vitro* modulation by micro-organisms will be examined.

Finally, an AMP named “paracentrin” could be candidate as anti-human biofilm pathogens as well as antifouling substances.

The heparin-histamine system in the phagocytic line of a tunicate: an ancient cell system equivalent to vertebrate mast cells?

F Cima
Department of Biology, University of Padua, Padua, Italy

In the compound ascidian *Botryllus schlosseri*, sentinel-cells were observed in the oral siphon where they play an immunosurvellance role in the opening of the pharynx. Their morphology, histochemical characteristics and ability to engulf test-particles are typical of hyaline amebocytes of the blood phagocytic line.

Histochemical and immunohistochemical studies at light and electron microscope reveal that, like in vertebrate mast cells, heparin and histamine co-localize inside the granules of this cell type and exposure to compound 48/80, a specific degranulating agent of vertebrate mast cells, leads to cell degranulation, suggesting that polyfunctional cells separated functions and competences among various specialized cell types of the innate immunity throughout the chordate evolution. Heparin and histamine were found in the temporary "plug" of colloidal matter that closed the oral siphon after 15 min exposure to bacterial spores in seawater, resulting by degranulation of the sentinel-cells. The main physiological functions of these substances is discussed. Heparin might be involved in the releasing activity of proteases, antimicrobial peptides, histamine, cytokines and growth factors. Histamine might be involved in the modulation of the ciliary beat in the pharynx. In short-term branchial cultures, exogenous histamine significantly increases the ciliary beat frequency and the presence of H2 receptors was indirectly demonstrated by means of the specific antagonist ranitidine suggesting a model of clearance similar to the mucociliary transport in the vertebrate respiratory tract.

Inflamed adult pharynx tissues and swimming larva of *Ciona intestinalis* share CiTNFα-producing cells

N Parrinello, A Vizzini, G Salerno, MA Sanfratello, M Cammarata, V Arizza, M Vazzana, D Parrinello
Department of Animal Biology, University of Palermo, Palermo, Italy

In situ hybridization and immunohistochemistry analyses showed that the *Ciona intestinalis* CiTNFα, previously cloned and sequenced, is both a component of the inflammatory pharynx response to lipopolisaccharide and is involved in swimming larva. Specific antibodies against a CiTNFα peptide identified a 43 kDa cell-bound form and observations of pharynx histological sections (at 4h and 8h p.i.), and in naïve and sham ascidians, disclosed the tissue response. Granulocytes with large granules and compartment/morula cells were CiTNFα-producing inflammatory hemocytes; the vessel endothelium was also involved in the response. Hemocyte nodules in the vessel lumen or associated with the endothelium showed the involvement of CiTNFα in producing lymphocyte-like cells in differentiating inflammatory hemocytes.

Finally, larva histological sections and whole mount preparations revealed that CiTNFα was expressed by trunk mesenchymal, preoral lobe, and tunic cells, suggesting mesenchyme immigration events and an ontogenetic role.

The hemocytes of *Polyandrocarpa misakiensis*, with particular reference to immunocytes

L Ballarin[1], K Kawamura[2]
[1]Department of Biology, University of Padua, Padua, Italy
[2]Laboratory of Cellular and Molecular Biotechnology, Faculty of Science, Kochi University, Kochi, Japan

Polyandrocarpa misakiensis is a polystyelid compound ascidian, common along the coasts of the temperate regions of Japan, which can reproduce asexually through continuous budding from parental zooids. The hemolymph of this species contains the following main cell-types: undifferentiated cells, phagocytes, including both hyaline amebocytes and macrophage-like cells, microgranular leukocytes, macrogranular leukocytes, morula cells and pigment cells.

Immunocytes are represented by phagocytes and morula cells. Phagocytes are capable of constitutive macropinocytosis, can release lectins and ingest macrogranular leukocytes in order to provide nutrients required for growth of developing buds. Like other compound ascidians, *Polyandrocarpa* morula cells (MCs) represent one of the most abundant circulating hemocyte types. They share the presence of DOPA-oxidase activity inside their vacuoles, ascribable to the presence of phenoloxidase, a key enzyme in invertebrate immune responses, which is released in extracellular compartment upon the recognition of foreign cells or molecules thus inducing a cytotoxic response. This enzyme can be considered, in all respects, a differentiation marker of morula cells as no other cell types show similar enzyme activity. Recently, we were able to clone a cDNA sequence of 1985 bp, representing most of the PO transcript. It shows homology with arthropod hemocyanins and, through *in situ* hybridization, we demonstrated that MC are the only cells expressing the corresponding mRNA.

Natural apoptosis during the blastogenetic cycle of the colonial ascidian *Botryllus schlosseri*

F Schiavon, L Manni, M Del Favero, L Ballarin
Department of Biology, University of Padua, Padua, Italy

Colonies of the compound ascidian *Botryllus schlosseri* undergo regular generation changes during which adult zooids are progressively resorbed and replaced by growing buds. The generation change, or take-over, is characterized by massive cell death by apoptosis, changes in the expression of surface molecules by senescent cells of zooid tissues and recruitment of circulating phagocytes in zooid tissues which assure the complete clearing of the dying cells. The entire process lasts 24-36 h at 20 °C and has been subdivided, on the basis of the degree of contraction of old zooids, in four substages.

It has an antero-posterior progression, at least in the digestive tube, as trace of apoptosis can be found in the epidermis, peribranchial epithelium, and

heart in the late take-over, whereas they are easily found in the branchial basket after 2-4 h from the beginning of the generation change.

During the take-over, massive recruitment of phagocytes, which ingest senescent cells, occurs: this suggests the release of diffusible chemoattracting factors by effete cells, able to attract phagocytes toward them.

We are going to investigate the expression of genes related to apoptosis, such as *IAP* (inhibitor of apoptosis) and *HSP70* during the various phases of the colonial blastogenetic cycle.

Session 4. Chairman: U Oreste CNR, Institute of Protein Biochemistry, Naples, Italy

The chick embryo as a hatching model for molecular biology of development and functional genomics studies

V Zappavigna
Department of Animal Biology, University of Modena and Reggio Emilia, Modena, Italy

The chick embryo has since long represented an ideal animal model for the study of development in vertebrates. It's easy accessibility for *in ovo* manipulation and it's relatively inexpensive handling have always represented an added bonus with respect to more conventional rodent animal models.

In recent years, the possibility to perform gene expression analysis and gain- and loss-of-function experiments, together with the completion of the first draft of the sequence of its genome, has made the chick one of the most versatile experimental systems available. Past and novel applications of the chick embryo model will be discussed, along with possible future developments of this effective *in vivo* system for fundamental and biomedical research.

Effects of marine toxins on *Xenopus laevis* early development

A Franchini, D Malagoli, E Ottaviani
Department of Animal Biology, University of Modena and Reggio Emilia, Modena, Italy

Okadaic acid (OA) is a lipophilic compound produced by several marine dinoflagellates, almost exclusively accumulated in mussel digestive gland. The consumption of contaminated animals provokes a syndrome in humans known as diarrheic shellfish poisoning. Palytoxin (PTX) is a large, water soluble polyalcohol found in a variety of marine organisms ranging from dinoflagellates to fish, implicated in seafood poisoning and classified as neurotoxin.

Our experiments were performed by using the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) protocol, and *X. laevis* embryos at early gastrula stage were treated with different toxin concentrations (0.1, 1 and 10 nM for OA and 0.37, 37 and 370 nM for PTX) for 5 days. The bioassay showed that both toxins affected *Xenopus* development in terms of mortality, delayed growth and embryo malformation. In particular significant mortality rates were observed with PTX higher dose and the initial sample population decreased by about 80 % at assay end. The observation of surviving larvae showed a marked tail folding. Further histological and histochemical studies revealed disorders to the nervous system (the most sensitive tissue) and to the tail skeletal musculature, while alterations also involved the main visceral organs.

Molecular biology-based experiments assessed the expression of four genes (*siamois*, *engrailed-2*, *bmp4*, and *myf5*) involved in the early events of *Xenopus* development (stages 11-47) and showed that PTX induces an increase in expression levels in all genes, while the response to OA is stage-dependent, with the embryonic development stages more sensitive than the larval stages. The de-regulated gene expression patterns may account for FETAX and histological data.

Regenerative capacity in *Xenopus laevis* tadpole tail

E Bertolotti, A Franchini
Department of Animal Biology, University of Modena and Reggio Emilia, Modena, Italy

The ability to regenerate lost tissue and organs varies among animal species, tissue and life cycle stages and the nature of repair responses has been related to the dynamic, reciprocal interactions among several signalling molecules and the cell types present and activated at the wound site. Amphibians, and in particular *Xenopus laevis,* provide excellent models to examine cellular and molecular mechanisms involved in the progressive loss of regenerative potential.

In this context, *X. laevis* tadpoles in different regenerative competence stages (st 50 and st 55) were studied after tail partial amputation. The histochemical results showed similar sequences of repair events, i.e., the epithelial wound closure, the formation of a neural ampulla and a bullet-shaped mass of cells at the cut end of the notochord surrounded by mesenchymal-like cells, but they were by means different morphological patterns. Moreover differences were in a delayed full tail reconstitution in st 55, and in the extent of inflammatory responses and angiogenesis at the wound tail stumps. Immunoreactivity to antibodies for critical healing immune mediators, such as inducible nitric oxide (NO) synthase, was also found in a greater number of cells, mainly leukocytes, from the early healing phase in older tadpoles.

On the whole, our data indicate an important involvement of the immune cells and induction of NO synthesis in wound microenvironment in modulating the degree of immune responses and repair quality outcomes that may be at least partly related to the gradual decline of tail regeneration efficiency during tadpole development.

***Bathyraja eatonii* immunoglobulin heavy chains**

V Avagliano, MR Coscia, S Varriale, E Chianelli, E Cocca, U Oreste
CNR, Institute of Protein Biochemistry, Naples, Italy

Chondroichthyes immunoglobulins (Ig) have been extensively studied in sharks; in skates investigations remain scarce and fragmentary. Only two heavy chain isotypes IgM and IgW (previously called IgX) have been reported in *Leucoraja erinacea* and *Raja eglanteria*; cDNA encoding the IgM membrane-bound immunoglobulin heavy chain has been sequenced only in *Leucoraja ericacea*.

To focus on Rajdae Igs we chose a cold adapted species, *Bathyraja eatonii* which lives in Antarctic seas at sub-zero temperature. We prepared mRNA from the spleen of an adult individual caught near the USA Palmer station. RT-PCR experiments were performed with two oligonucleotides designed on the Rajdae sequences available: the first, used as sense primer, in the FR3 region of the variable domain, the second, used as anti-sense primer at the end of the CH4 domain, including the stop signal. The PCR products were analysed on agarose gel and found to be homogeniously 1400-nt long. They were cloned in the pSC-A vector and 11 positive clones were sequenced.

The sequences shared on average 97.3 % nucleotide identity. By Shannon entropy analysis, the highest position diversity was found in the CDR3 region, whose length varied between 8 and 12 amino acid residues. A distance tree was built by the Neighbor-joining method and two indipendent branches, defining two IgM subisotypes, were obtained.

Based on comparison of the *B. eatonii* amino acid sequence with that of *L. erinacea* we found one additional cystein residue in the CH3 domain and same number of glycosilation sites.

Evidence for local production of immunoglobulins in the skin of Antarctic teleosts

E Chianelli[1], C Motta[2], S Giacomelli[3], MR Coscia[1], S Varriale[1,4], V Avagliano[1], U Oreste[1]
[1]CNR, Institute of Protein Biochemistry, Naples, Italy
[2]Department of Evolutive and Comparative Biology, University of Naples "Federico II", Naples, Italy
[3]Zoological Station "Anton Dohrn", Naples, Italy
[4]Department of Chemistry, University of Naples "Federico II", Naples, Italy

The skin of teleost fishes has an important immunological role against pathogens, similarly to other tissues such as the gastrointestinal, respiratory, and genitourinary tracts. It has been reported that skin and gut mucus of teleost fishes contain tetrameric IgM, that are produced independently of the systemic antibodies, thus indicating the existence of a mucosal immune system in teleosts.

Secretory immunoglobulins (Ig) similar in size to serum Ig, have been purified from the skin mucus of the Antarctic teleost *Trematomus bernacchii*. Lymphocytes that produce Ig have been localized in the skin tissue by immunohistochemistry, using specific antisera, and by *in situ* hybridization.

Skin sections were analyzed to verify the expression and synthesis of IgM and localize the cells involved. By *in situ* hybridization using an anti-sense probe, the expression of both L and H chain genes was demonstrated in occasional lymphocytes located close to the basal membrane as well as dispersed in the superficial stratum. By contrast, filamentous cells and goblets cells showed no specific labelling. No signal was detected in any of the above mentioned cells when hybridization was carried out using a sense probe.

Moreover, RT-PCR experiments performed using specific primers revealed the presence of mature transcripts encoding the secretory Ig in the skin of *T. bernacchii* and that of the membrane-bound form in the case of *Chionodraco hamatus.*

The *ζ-ζ* CD3 component of the TCR complex in lipid bilayers

S Varriale[1,2], A Merlino[2], V Avagliano[1], MR Coscia[1], E Chianelli[1], L Mazzarella[2], U Oreste[1]
[1]CNR, Institute of Protein Biochemistry, Naples , Italy
[2]Department of Chemistry, University of Naples "Federico II", Naples, Italy

The human T-lymphocytes receptor complex consists of the antigenic peptide binding subunit, the heterodimer *α-β*, and the transducing subunit CD3, the latter resulting from the assembly of three dimers: γ-δ, γ-ε and ζ-ζ. While the structure of the extracellular portions of *α-β*, *γ-δ* and *γ-ε* has been determined, that of the regions traversing the cell membrane (TM) are unresolved with the exception of ζ-ζ. In fact by NMR spectroscopy in water, the structure of *ζ-ζ* TM region has been recently proposed. The present work has been aimed at analysing the human homodimer TM structure in lipid bilayers by Molecular Dynamic simulations.

We considered two types of lipid bilayer models: the palmitoyl-oleoyl-phosphatidylcholine (POPC) which better resembles the cell membrane in lipid composition, and the POPC/cholesterol/palmitoyl-sphyngomyelin (1:1:1) which resembles the raft membrane microdomains, thought to be the sites of the signal transducing machinery.

The simulations were performed using the GROMACS package for a total time longer than 25 ns. Each simulation showed the formation of a stable homodimer with high conservation of the secondary structure. The model in raft had an a-helix structure more extended (for each chain: 6.3-17.0 Å in raft and 12.3-27.4 Å in POPC); a more compact packing (the distance between the centers of mass of the two helices was 7.9 Å in raft and 9.7 Å in POPC).

Our results suggest that during the translocation of the TCR complex in the raft membrane domains, the *ζ-ζ* dimer assumes a specific conformation probably necessary to the correct signal transduction.

Molecular and structural characterisation of a macrophage migration inhibitory factor from sea bass (*Dicentrarchus labrax*)

E Randelli[1], F Buonocore[1], AM Facchiano[2] , A Pallavicini[3], M Modonut[3], G Scapigliati[1]

[1]*Department of Environmental Sciences, Tuscia University, Viterbo, Italy*
[2]*CNR, Institute of Food Sciences, Avellino, Italy*
[3]*Department of Life Science, University of Trieste, Trieste, Italy*

The macrophage migration inhibitory factor (MIF) is a cytokine mainly produced by T lymphocytes and macrophages in response to inflammatory stimuli. MIF is also implicated in a wide range of biological activities, related to hormone-like and enzymatic functions.

We report here the identification, from a thymus cDNA library, of a cDNA encoding a MIF molecule from sea bass (*Dicentrarchus labrax*), the transcription levels, and the 3D structure.

Sea bass MIF cDNA consists of 609 bp and encodes for a putative protein of 115 amino acids. Real time PCR analyses revealed that MIF is constitutively expressed in various tissues and organs, with the highest mRNA level observed in thymus. MIF expression was induced after 4 hours *in vitro* stimulation of HK leukocytes with LPS and decreased after 24 h. The predicted 3D model of sea bass MIF is comparable with human model and has been used to verify the presence of structural requirements for its known biological activities in mammals.

Our results will raise the possibility of investigating more in detail the involvement of MIF in sea bass immune responses and add new insight on the evolution and biological activities of this important cytokine present both in vertebrates and invertebrates.

Immunomodulatory effects of *Aloe arborescens* ethanolic extract on SAF-1 cell line

C Bernini[1], C Belardinelli[1], E Ovidi[1], D Ceccarelli[1], F Buonocore[1], L Abelli[2], G Scapigliati[1], AM Fausto[1], S Picchietti[1]
[1]*Department of Environmental Sciences, Tuscia University, Viterbo, Italy*
[2]*Department of Biology and Evolution, Section Comparative Anatomy, Ferrara University, Ferrara, Italy*

Modulation of cytokine secretion may offer novel approaches in the treatment of a variety of fish diseases. The use of herbal extracts can represent a new strategy for the modulation of cytokine expression. Deeply studied cytokines are interferons (IFNs), which may induce vertebrate cells into an antiviral state.

The *in vitro* research performed in this study demonstrated that an ethanolic extract (1.2 mg/ml) of *Aloe arborescens*, a plant that is widely used in Korea as ingredient of health food and cosmetics, significantly increased after 48 h of treatment the expression level of the IFN type I in SAF-1 (*Sparus aurata* fibroblast cell line) cells stimulated with Poly I:C. Moreover, the treatment significantly up-regulated (after 48 h) the expression levels of MHC class I-α and Mx, a protein endowed with antiviral properties, compared with control cells. This work provides a new perspective for the use of medicinal plants in fish to prevent viral diseases. Further studies are in progress to characterize the active principles of the plant extract and to establish feeding protocols for food additives.