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Report of Meeting

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Organizers: **M Betti, M Balsamo, S Papa**

Department of Human, Environmental and Natural Sciences, University of Urbino "Carlo Bo", Urbino, Italy

Session 1

1999-2009: a 10-year apprenticeship in comparative immunology. The immunocytes as leading actors in the responses of the bivalve *Mytilus* to environmental challenge

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In bivalve molluscs, circulating hemocytes are responsible for the innate immune defence, and are also involved in the transport of nutrients from the digestive gland to the gonad during gametogenesis. Although it has long been known that the activity of these cells can be modulated by multiple signals, only in recent years the mechanisms involved in the responses of hemocytes to different stimuli, from both the internal and the external environment, have been investigated.

In the marine bivalve, the edible mussel *Mytilus*, studies carried out both *in vitro* and *in vivo* on the effects of challenge with different bacterial species and strains, heterologous cytokines, as well as with a number of environmental contaminants, lead to the identification of the main signaling pathways involved in immune activation, as well as of the main mechanisms of cellular damage and consequent immunodepression. Moreover, endogenous estrogens were identified as physiological modulators of the hemocyte function. The results obtained added force to the hypothesis that the mechanisms of innate immunity are extremely conserved between invertebrate and mammalian systems.

Overall, the data collected so far allowed the functional characterization of mussel hemocytes as a model for investigating both the basic processes of the immune response, and the mechanisms toxicity of emerging environmental contaminants. Moreover, these studies demonstrated that *Mytilus* hemocytes represent a sensitive target for environmental stimuli, and that changes in their function are related to significant changes in the physiological status of bivalves, an ecologically and economically relevant group of invertebrates.

Effects of bacterial challenge on *Mytilus* digestive gland biomarkers

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In bivalve molluscs, responses to bacterial challenge have been largely investigated in the immune cells, the hemocytes, whereas little is known about the possible effects at the tissue level. In this work, the effects of *in vivo* challenge of mussels (*Mytilus spp.*) with different bacteria (*Micrococcus lysodeikticus*, *Vibrio anguillarum*, *Vibrio splendidus*) on different biomarkers of stress were evaluated in the digestive gland at different times post injection (p.i.).

All bacteria induced a significant decrease in lysosomal membrane stability - LMS (about -50 %) with respect to controls (PBS-NaCl-injected mussels) from 3 to 24 h p.i. When expression of antioxidant genes was evaluated by quantitative RT-PCR with both *M. lysodeikticus* and *V. anguillarum* a general downregulation of both metallothionein isoforms, MT10 and MT20, was observed, with the exception of a large increase in MT20 mRNA induced by *V. anguillarum* and a smaller increase in MT10 by *M. lysodeikticus* at 6 h p.i. Both bacteria downregulated glutathione S-transferase - GST-π at all time p.i. *V. anguillarum* decreased the expression of catalase, wherease no effects were observed with *M. lysodeikticus*. At 48 h p.i. the activity of

catalase was increased (2-fold with both *M. lysodeikticus* and *V. anguillarum*; +60 % with *V. splendidus*) and total GST activity was decreased (-30, -40 %) with all bacteria. Both *M. lysodeikticus* and *V. splendidus* also decreased GSH reductase - GSR activity (about -24 %) and total glutathione content (-47 %) whereas no effects were observed with *V. anguillarum*.

Flow cytometry as a tool for analyzing invertebrate immunocytes: our experience with *Mytilus* hemocytes

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Flow cytometry (FC) represents a powerful tool generally utilized in biomedical research. FC has been also increasingly applied in invertebrate immunology; in particular, in bivalve molluscs, FC has been largely utilized to characterize hemocyte populations and to evaluate total cell count and oxyradical production. In addition, FC can be employed to recognize antigens, or at least immunodominant epitopes, shared in common with mammalian cells, including human leukocytes.

In addition to traditional methods such as microscopy and protein chemistry, FC can provide a simple, reproducible, and sensitive method for evaluating invertebrate hemocyte responses to different immunological stimuli.

Data are here summarized obtained in different *in vitro* and *in vivo* studies carried out on the hemocytes of the marine bivalve *Mytilus galloprovincialis*. FC was successfully utilised to evidentiate, utilizing annexin V binding and mitochondrial markers, pre-apoptotic processes in mussel hemocytes in response to cytokines (TNFα) and emerging contaminants (nanoparticles), as well as proliferation/differentiation processes in response to growth factors (Stem Cell Factor-SCF). Furthermore, we have recently investigated the presence of different antigens (CD34, CD117 and CD11b) in mussel immunocytes and changes in their expression following in vivo challenge with both Gram(+) bacteria and autoctonous vibrio species.

These data support the importance of developing the utilization of FC in comparative and environmental studies on invertebrate immunocytes.

New knowledge of antimicrobial peptides in Mediterranean mussel

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Marine invertebrates are constantly surrounded by potentially invading microorganisms. Their defence mechanisms involved circulating haemocytes that infiltrate injured tissues, encapsulate or phagocyte microbial cells and release cytotoxic factors such as lectins, complement factors and antimicrobial peptides (AMPs). Mussels especially seem more resistant to infections and diseases than other edible bivalves.

In the frame of the *European Integrated Project FOOD-CT-2005-007103*, EST production and gene expression profiling in *M. galloprovincialis* (Lmk., 1919) are providing new interesting evidences.

Massive EST sequencing of primary cDNA and normalized libraries of immuno-stimulated mussels revealed a high sequence variability of AMPs, especially in haemolymph. These results confirm the importance of such molecules in innate immune response of marine invertebrates.

Selected immune-specific and immune-related trancripts were arrayed in the first mussel *Immuno-oligochip*. Preliminaries microarray and real time experiments showed various gene expression changes in mussels injected with bacterial antigens.

Moreover, the new generation sequencing technologies provide us plentiful and precise knowledge of the variability of these natural antibiotics, that can explicate better the ancient host-pathogen interactions and the adaptation strategies.

Proliferation of mussel haemocytes: effects of Stem Cell Factor (SCF)

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Stem Cell Factor (SCF) is a member of mammalian haematopoietic cytokines, a group of glycoproteins that regulate the growth and differentiation of haematopoietic cells and functionally activate mature neutrophils or macrophages. In bivalve molluscs, circulating haemocytes resemble the monocyte/macrophage lineage. However, in these organisms, no information is available on the physiological control of haematopoiesis. In this work, the *in vitro* effects of heterologous SCF on the haemocytes of *Mytilus* sp. were investigated.

Flow cytometry (FC) analysis of control haemocytes identified 3 main cell subpopulations: granulocytes, agranulocytes, blast-like cells. A significant proportion of total haemocytes showed immunoreactivity towards anti-CD34 and anti-CD90 antibodies, utilised as markers of haematopoietic stem cells.

SCF induced significant changes in haemocytes functional parameters, indicating increase in phagocytic activity and reduction in lysosomal membrane fusion processes. Moreover, FC analysis showed that SCF significantly affected

both haemocyte number and cell cycle, mitochondrial activity, and immunoreactivity towards different anti-CD-antibodies. In particular, increases in the number of granular (phagocytic) haemocytes were observed; the effects of SCF involved the activation of c-kit tyrosine kinase-like receptors.

Ultrastructural morphological studies in colcemide-treated haemocytes confirmed that the large majority of cells are able to enter the mitotic phase of the cell cycle.

The results support the hypothesis that common pathways involved in modulating activity, proliferation and differentiation of immune cells are conserved from molluscs to mammals.

Effects of the protein pheromone E*r*-1 isolated from the ciliate *Euplotes raikovi*, on the phagocytic activity of the bivalve *Mytilus galloprovincialis*

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Phagocytosis plays a central role in the cell-mediated immune response and the study of cytokine-like molecules capable of modulating the phagocytic activity of immunocytes may contribute insightful information on the evolution of the immune response. Previous experiments on the immunocytes of the mussel *Mytilus galloprovincialis* suggested that regulation of the phagocytic activity involves cAMP- and PKA-pathways. We have now analyzed the response of these immunocytes to the effects of a protein pheromone, denoted E*r*-1, produced by the protozoan ciliate *Euplotes raikovi* and characterized by an exclusively helical structure like IL-2 and its cytokine family members. Our results indicate that E*r*-1 increases the immunocyte phagocytic activity, and this increase follows cAMP- and PKA-dependent signal transduction pathways. This indication is consistent with the activity of ciliate pheromones in mechanisms of self-nonself recognition and cell-cell adhesion.

***Mytilus* hemocytes as a model for nanoparticle toxicology in marine invertebrates**

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The potential for human and ecological toxicity associated with nanomaterials is a growing area of investigation. In mammalian cells, nanoparticles (NPs) have been shown to induce inflammation and oxidative stress, and changes in cell signalling and gene expression. As the nanotechnology industries increase production, nanoscale products and by products will enter the aquatic environment, posing a possible threat to aquatic organisms. In particular, filter-feeding invertebrates may represent a unique target group for nanoparticle toxicology, since they have highly developed processes for the cellular internalisation of nano- and microscale particles, endocytosis and phagocytosis, that are integral to key physiological functions such as intracellular digestion and cellular immunity. In this work we show that in the hemocytes of the marine bivalve *Mytilus* different types of engineered nanoparticles (Carbon black, C60 fullerene, TiO_2, SiO_2) induce activation of different immune parameters (lysozyme release, activation of oxidative burst and NO production) to a different extent depending on the NP type and concentration, without significant cytotoxicity. Only at higher concentrations mitochondrial damage was observed, this indicating pre-apoptotic processes. The inflammatory effects of NPs were mediated by rapid activation of the stress activated p38 MAPK. The results indicate that bivalve immunocytes represent a suitable model for investigating the potential effects of NPs in aquatic organisms.

Session2

Are really males the sterner sex? The immune responses of the clam *Tapes philippinarum* as a case study

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For the first time, gender-related differences in immune responses of the clam *Tapes philippinarum* were investigated. Haemocytes from male, female and sexually undifferentiated clams were collected, and total haemocyte count (THC), haemocyte volume, capability of haemocytes to assume the vital dye Neutral Red (indicative of endocytotic activity), acid phosphatase and lysozyme-like activities in both haemocyte lysate (HL) and cell-free haemolymph (CFH), were evaluated.

No statistically significant differences in THC values were observed. However, differing haemocyte size frequency distribution was found: the fraction of larger haemocytes (6-8 µm diameter, 200 fl volume) markedly increased in females, whereas the fraction of smaller haemocytes (< 5 µm diameter, < 200 fl volume) increased in both male and undifferentiated clams. Significantly increased Neutral Red uptake was recorded in haemocytes from females. This was most likely related to the higher fraction of larger haemocytes in females, these cells being more actively involved in phagocytosis. No significant variations in lysozyme-like activity was observed in HL, whereas in CFH enzyme activity resulted significantly higher in females with respect to male and undifferentiated animals. HL acid phosphatase activity was significantly higher in males with respect to females and undifferentiated clams, whereas no significant variations in enzyme activity was observed in CFH. Overall, results obtained demonstrated that gender-

related differences in immune responses occurred in *T. philippinarum*, and indicated that females had more active haemocytes than both male and undifferentiated clams, at least on the basis of the cellular parameters investigated.

Rhamnose-binding lectins in the compound ascidian *Botryllus schlosseri* as multifaceted immune molecules

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Rhamnose-binding lectins are characterised by the presence of common aminoacid motifs (YGR, DPC, KYL) and the presence of eight conserved cysteine residues involved in four disulphide bridges responsible of the compact structure of the proteins. They have been described in many fish and few invertebrates. Recently, in the compound ascidian *Botryllus schlosseri*, we identified and characterised a rhamnose-binding lectin (Bs-RBL) with opsonic activity, able to enhance phagocytosis of foreign target cells.

In the present work, we continued our investigation on Bs-RBL and studied the expression of the molecule through immunohistochemical and immunocytochemical analysis, using specific polyclonal antibodies, and *in situ* hybridisation on both colony sections and haemocyte monolayers.

Results obtained indicate the phagocytes as the site of synthesis of this kind of molecules as both the protein and the corresponding mRNA were located in this cell type. With immunoblot analysis of colony lysates, we studied the expression of Bs-RBL during the colonial blastogenetic cycle. The electrophoretic bands recognised by the anti-BsRBL antibody were of higher intensity during the cyclical colony generation change (take-over), characterised by massive apoptosis of zooid tissues, In addition, immunocytochemical analysis showed that that the frequency of haemocyte recognised by the anti-Bs-RBL antibody significantly increased during the take-over, and that, in addition to phagocytes with a labelled cytoplasm, the plasma membrane of other cell types (e.g., morula cells) resulted immunopositive, indicating that this molecule can recognise sugars on the surface of senescent cells, unexposed in healthy cells, and suggesting its involvement in efferocytosis.

The incubation of haemocyte monolayers with purified Bs-RBL results in the release in the culture medium of molecules recognised by antibodies raised against mammalian IL-1α and TNFα, we have already demonstrated to have an immunomodulatory role. As these molecules are synthesised and released by cytotoxic morula cells (MC), the above observation indicate an immunomodulatory role of Bs-RBL on MC activity.

In addition, it has been recently demonstrated that fish RBL can recognise LPS and lipoteichoic acid, basic components of Gram(–) and Gram(+) bacterial cell wall, respectively, suggesting an antibacterial role of these proteins. We are carrying out new experiments to verify this aspect in Bs-RBL.

Evolutionary analyses of sequence and intron-exon structure of two lectin genes in ascidians

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Lectins are a group of sugar-binding proteins that recognize specific carbohydrate structures and agglutinate cells by binding to cell-surface glycoconjugates. These proteins are involved in a variety of distinct role in different cells and species, and in invertebrates they are considered as recognition molecules that trigger defensive activities. Lectins show a wide variety of protein architecture, thus they are classified into several families depending on the sugar-binding specificities and the sequence similarities of the Carbohydrate-Recognition Domain (CRD). We have analysed the evolutionary history of the CRD of two lectin genes, the galectin and the Rhamnose-Binding Lectin (RBL), within chordates. In particular, these genes have been identified in the publicly available genomic sequences of *Ciona*, *Oikopleura* (Tunicata) and *Branchistoma* (Cephalochordata), all protochordate species, and have been annotated using several bioinformatics methods and also with the help of EST data. The evolutionary analyses have been carried out in a wide taxonomic sample including the homologous functionally characterized sequences of other deuterostome species, and the human annotated homologs. In addition to the traditional phylogenetic reconstructions based on amino acid sequences, we have also investigated the conservation of the intron-exon structure and evaluated the congruency between the results provided by these two different data types. Our results show that the in most cases the single CRDs are delimited by introns, with linkers and additional protein domains encoded by distinct exons, and that tunicate genes evolve always very fast both at level of sequence and gene structure.

Individuation of a new metallothionein from the urochordate *Ciona intestinalis*

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Metallothioneins (MTs) are metal-chelating proteins occurring in animals, plants and prokaryotes, involved in detosification and immunity. They do not constitute a monophyletic protein family, but rather a superfamily of heterogeneous, low molecular weight, cysteine-rich peptides.

Current knowledge on MTs comes mainly from vertebrate molecules which are composed of approximately 60 amino acids, including 20 cysteine residues, and folded into two independent domains when coordinating divalent metal ions.

Up to now, there are no descriptions of MTs in invertebrate Chordates althought it seems that the vertebrate structure is maintained also in other deuterostomes such as the echinoderms.

In this study, we present some data on MTs of the solitary urochordate *Ciona intestinalis*. We have cloned the transcript and characterized the gene of a new MT, codifying for 39 amino acids, including 12 cys residues (30 % of total amino acids, in accordance with other MTs); moreover, the typical organization of cysteine residues in C-X-C motifs is conserved. The gene is composed of two introns (one inside the coding region and the other inside the 3' UTR region) and three exons. The 5' untrascribed region contains several cis elements similar to those found in vertebrate MT genes such as: metal responding elements (MRE), antioxidant responding elements and STAT3, which are involved in constitutive and metal-related, ROS and cytochines induction, respectively. The amino acid sequence of *C. intestinalis* MT shows only limited similarity with other MTs, e.g., *Mytilus edulis* MT (28.8 % identity), *Strongylocentrotus purpuratus* MT (23.4 % identity) and *Sparus aurata* MT (36.7 % identity). Phylogenetic analyses with various methods are in progress.

A preliminary study by Atomic Force Microscopy of cytogenetic stability in a MDV-integrated chicken lymphoblastoid cell line

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In previous works we have demonstrated that the chicken lymphoblastoid T cell line MDCC-MSB1 is able to produce ovotransferrin and nitric oxide to a higher extent than CEF (Chicken Embryo Fibroblasts). This ability can be tentatively related to the integration of Marek's Disease Virus (MDV) genome. In addition, it is well known from literature that lymphocytes infected by MDV produce vIL-8, with a chemotactic function towards other lymphocytes, to amplify viral spreading. On the other hand, it is also known that the virus undergoes a fragmentation prior to a random integration, preferentially in subtelomeric regions. The viral integration may induce a novel organization of chromatin architecture, with a modified gene expression. In our opinion it is worthwhile trying to relate cytogenetic stability to functional modifications.

Recently, Atomic Force Microscopy (AFM) technique was applied to study the structure of chromosomes at nanoscale level. Structural analysis at high resolution of high molecular complexes provide detailed information such as 3-dimensional topological data, mechanical behaviours, dynamic processes and molecular interactions. When scanning soft biological surfaces, the AFM can achieve a resolution of about 1 nm. The vertical resolution is mostly determined by the AFM scanner sensitivity, and typically is 0.01 nm. These features allow to investigate the different structure of chromatin in the regions of the viral insertion. On the basis of these data, the correlation between the localization of viral insertion and the different gene expression due to Marek's Disease Virus localization is investigated.

Session 3

Self/nonself recognition in the ciliated protozoa: characterization of the pheromone gene family of *Euplotes nobilii*

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A family of seven allelic genes encoding self/nonself signal proteins (pheromones) in the polar (cold-adapted) marine hypotrichous ciliate, *Euplotes nobilii*, were cloned from the somatic sub-chromosomic genome of the cell macronucleus. The determination of their full-length nucleotide sequences shows that their open reading frames specify proteins of 83 to 94 amino acid residues which represent the cytoplasmic pheromone precursors (pre-pro-pheromones). Two proteolytic steps would thus remove the pre and pro segments formed by tightly conserved sequences, before the secretion of the structurally more variable mature proteins. At odds with respect to the general structural organization of the macronuclear genes of the hypotrichous ciliates, the 5' region of all the cloned pheromone genes is significantly longer than the respective coding region (approximately, 350 *versus* 250 nucleotides). Considered jointly with the tight sequence conservation, this feature implies that the 5' region is site of specific activities in the mechanism of pheromone gene expression.

Antimicrobial peptide transcription is modulated after repeated exposure to heat-inactivated *Escherichia coli* in *Drosophila melanogaster* SL2 cell line

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Recent findings in *Drosophila melanogaster* indicate that the activation of the immune system immediately before an infection increases the resistance to pathogens, suggesting the possibility that a first exposure to an immunogen may strengthen the immune response to a second challenge. By RT-PCR experiments, we have observed in the *D. melanogaster* SL-2 hemocyte cell line that a second 6 h exposure to heat inactivated *Escherichia coli* significantly increases the expression of the antimicrobial peptides (AMPs) drosomycin and diptericin, with respect to the first 24 h exposure. Conversely, expression levels reached after the first 24 h exposure are not exceeded after a second 6 h immune challenge by the AMPs defensin and cecropin A1 and by the putative helical cytokine, DHF. Surprisingly, the expression of the Imd-related kinase dTAK1, is increased by the first incubation with bacteria, while its expression is lower than in controls after the second exposure. The augmented expression

observed for drosomycin and diptericin is the consequence of the additive effect of the second exposure. In accordance with data present in literature, our observations suggest the existence in *D. melanogaster* of mechanisms devoted to control the intensity of the immune response. This regulation involves the possibility to modulate the expression of AMPs, in our case drosomycin and diptericin, and signal transduction regulators, e.g., dTAK1.

Effects of bacterial injection and salinity stress on the humoral immune response of *Paracentrotus lividus* (Echinodermata)

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The altered expression of iNOS and lysozyme in the coelomocytes present in the wall of the gut of *P. lividus,* was observed after bacterial injection and osmotic stress by histochemical and immunocytochemical procedures.

After 3 h from injection of bacteria a significant increase expression of iNOS was observed. This increase remained at the same level until 24 h post inoculum. Conversely, the expression of lysozyme in the coelomocytes showed a relevant increase only after 24 h p.i.

We also observed that the expression of iNOS and lysozyme after osmotic stress increased following the pattern registered after bacterial injection.

In accordance with data present in literature, the present study identifies the coelomocytes of the sea urchin as the main effectors of defense response and indicate that the expression of iNOS increases faster than that of the lysozyme.

Does *Trichoplax* (Placozoa) produce resistance stages?

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Trichoplax adhaerens (Placozoa) is currently regarded as the most primitive living metazoan. Its mitochondrial and nuclear genomes have been wholly sequenced but its life cycle is still little known. Placozoa reproduce asexually, but molecular evidences suggest the occurring of sexual events. Under stress conditions, animals enter a degenerative phase (D-phase), and produce few, large oocytes. The existence of spermatozoa has not yet proved with certainty. The D-phase was induced in laboratory cultures of *Trichoplax*, and after four weeks the production of oocytes was observed *in vivo*. After at least another week eggs were released through animal body disgregation. Animals containing oocytes, and isolated eggs at 5, 6 and 10 weeks of age were studied under optical microscopy, SEM and TEM. The oocyte in the animal body at first shows only the oolemma, then produces an external envelope made up of 2-3 layers of different thickness and nature: at that stage it is released. Early stages of cleavage were occasionally seen, in rare cases even in the animal body. Very few isolated eggs in cleavage phase and many undivided ones were observed in the cultures: all showed an envelope with an identical morphology. That suggests that sexual reproduction is performed only in adverse environmental conditions, and that both quick-hatching and late-hatching eggs might be produced. The formation process of the thick egg envelope and its fine structure appear very close to those reported for the asexual cysts of some Ciliata. That supports a possible resistance function of the *Trichoplax* eggs to stress conditions like those induced in the cultures, or those occurring in natural unstable habitats. *Trichoplax* lives as an epibenthic form in the littoral marine environment, where the ecological conditions are quite variable and resistance stages are produced by a number of colonizing organisms (e.g., cysts of protists, gemmules of sponges).

Identification of a functional motif mobilized by retroelements in mRNA from activated human lymphocytes

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3' untranslated regions (3' UTRs) of eukaryotic mRNAs commonly contain conserved repeated motifs involved in the control of mRNA stability, i.e., through micro-RNA (miRNA) mediated regulation. Following alignment of functionally related genes (i.e., of genes co-regulated in response to the same stimuli), we identified a number of conserved motives shared by genes involved in the same process (in particular: lymphocyte activation). Primers were designed on these putative regulatory motives and used for RT-PCR fingerprintings on cDNA from human T lymphocytes before and after PHA pulse and IL-2 activation. We noticed complex patterns of bands only when using cDNA from activated cells. Bands were cloned and sequenced. Quantitative RT-PCR confirmed that the identified genes, containing the putative regulatory motives, were expressed only in activated cells. Searches in miRNA databases (miRBase) showed that one of our motives, CACTN (3,4,3), corresponds to a sequence of a known miRNA family, and thus confirmed its regulatory role. This motif, highly conserved across evolution, in ruminants is part of a Short Interspersed Nuclear Element (SINE) called Bov-A2. This SINE is integrated in regulatory regions (promoter, 5' and 3' UTR, introns) of many immune-related genes. These results are suggestive of connections among retroelements and the sharing of common regulative motives among functionally related genes. In particular, the role of retroelements could be to carry these important motives and help in their spreading among genes (seed) as already suggested by several authors.

Our results, in turn, suggest a deep involvement of these processes in immunity and in the regulation of genes involved in the response of immune subsets like lymphocytes to external stimuli.

Session 4

An attempt to re-examine the immune role of *Ciona intestinalis* hemocytes

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In the last few years, interest in the mutual relationships between ascidians hemocytes and products of innate immunity gene repertoire has led to a more clear-cut knowledge of multifunctional role of hemocytes in *Ciona intestinalis* immunity. This is a suitable approach that may include morpho-functional screening methods allowing us to disclose differentiation of the hemocytes, their activities in immune responses leading to a more precise and reasonable classification taking in account a multi-parameter approach. The genome sequence provided new insight in studying the innate immunity. Bioinformatic approach and extensive *in silico* search have concerned immunorelevant molecules, gene expression patterns and some specific immune properties that contribute to clarify morpho-functional aspects.

The involvement in immunity of hemoblasts, lymphocyte-like cells, hyaline amebocytes, and various types of granulocytes have been described.

The *Ciona intestinalis* prophenoloxidase activating system during LPS inflammatory reaction

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Phenoloxidases initiate melanin synthesis in almost all organisms, and are involved in different biological activities. In many invertebrates, defense reactions are linked to phenoloxidase activity and/or melanization, an innate response generally observed in wounded tissues. Contact with a foreign molecule is able to trigger the prophenoloxidase (proPO) system that requires serine protease cleavage for activating the zymogen to phenoloxidase (PO). It is generally accepted that the proPO system is fully expressed in arthropods, and, recently, progress in the regulation of crustacean and insect proPO activation steps has been made. After cells are stimulated by components of the pathogen associated molecular pattern (PAMP), proPO activation takes place via zimogenic serine proteinase in turn activated by PAMP. In the present paper, we report on the *Ciona intestinalis* proPO system and related molecules, with particular focus on the biochemical, cellular and molecular components of the proPO system in the tunic tissue following LPS intratunic injection. Tunic homogenate supernatant (THS), assayed with the Dopa-MBTH reaction, displayed Ca^{2+}-independent PO activity that was increased by LPS and further enhanced by proteases. In vivo experiments were performed by injecting isosmotic medium or LPS, and THS was assayed for its PO activity. To determine the PO response at the injured site, an assay with Dopa-MBTH was performed *in vitro*. Quinones were mainly contained in the tunic matrix area enriched with inflammatory cells around the injection site. Microscopy observations and immunohistochemistry with anti-CinPO2 antibodies showed granulocytes and unilocular refractile granulocytes containing PO. Immunoblotting with anti-CinPO2 and SDS-PAGE zymograms demonstrated PO activity linked to different bands as an effect of LPS injection. These POs distinguishable by their size are contained and presumably released by tunic inflammatory cells and hemocytes of the pharynx bars.

Proliferating and proPO activity of the hemocytes of *Ciona intestinalis*

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Hemocytes are essential in Tunicate immunity, performing functions such as phagocytosis, encapsulation and lysis of foreign cells. Because of the *Ciona intestinalis* phylogenetic taxonomic position between invertebrate and vertebrate, in the last decades, a molecular biology approach has been used to identify the gene expressed in the hemocytes of this Tunicate. Here, we tried to throw light on hematopoiesis in *C. intestinalis*. Therefore, we used circulating hemocytes, as source of Hpt stem cells, and separated them by discontinuous percoll gradient. We characterized the hemocytes with a blood cell staining method, such as MayGrunwald-Giemsa staining. Then we cloned 2 predicted hemocyte genes: PCNA, as marker to identify Hpt stem cells and their progenitors; proPO2, as marker for differentiation and for studying a well known innate defense mechanism of the hemocytes of *C. intestinalis*. We valued the expression for both the genes by RT-PCR, and for PCNA gene, by ISH. We made primary culture attempts of the circulating hemocytes, and valued their vitality and proliferating activity by MTT assay. The results of the RT-PCR, showed that, the hemocytes of the percoll second band expressed more than those of the other bands both PCNA and proPO2 genes, whereas, by ISH, the transcript for PCNA gene was present in 4 of all the hemocyte types: hyaline and granular amoebocytes, URGs and lymphocyte-like cells. The primary culture attempts showed that the hemocytes of the second band seem to proliferate, arrange and form clumps

already 30 min after plating, and that these formations become brown within 24 h.

Session 5

Characterisation of a progranulin in the medicinal leech, *Hirudo medicinalis*

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Progranulin (PGRN) is a pluripotent growth factor expressed in many animal groups and involved in development, tumorigenesis, inflammation and wound repair. Its mechanisms of action remain largely unknown. In animals, the progranulin gene encodes a cystein rich glycoprotein containing several repeated motifs called granulins (GRN). Granulins are 6 kDa peptides produced by proteolytic cleavage of the precursor PGRN. In vertebrates, PGRN is expressed in leucocytes and is involved in defence as well as in wound healing events. Its processing is linked to regulation mechanisms of inflammation.

To better understand the function(s) of (pro)granulin, we investigated the role of this molecule in an invertebrate model, the medicinal leech *Hirudo medicinalis*. The *Hirudo* progranulin (*HmPGRN*) gene codes a 150 kDa protein containing 16.5 putative GRN motifs and showing a high similarity with human and other animal PGRN. *Hm*PGRN is constitutively expressed in leech body and 24 hours after injury the protein accumulates as granules in granulocytes, belonging to fibrovascular tissue (a peculiar hirudinean tissue). These cells, after surgical lesion, increase in number and migrate towards the wound site.

In central nervous system (CNS) of mammals this molecule is expressed by neurons and microglial cells and probably acts as a neurotrophic factor. In human, mutations of PGRN gene are linked to frontotemporal lobar degeneration and other neurodegenerative pathologies. In leech CNS, *HmPGRN* gene is constitutively expressed in neurons and is upregulated during nerve cord repair. Immunohistochemical analysis revealed the accumulation of the protein in neurons cell bodies after injury. Taken together, these data suggest that *Hm*PGRN might play a neurotrophic role and contribute to the repair process. Since progranulin structure is highly conserved in animals, its study in leech can increase our knowledge on its functions in normal or injured tissues and lead to new therapeutic investigations.

Session 6

Cellular and molecular responses of the teleost fish *Dicentrarchus labrax* against *in vivo* challenge with nodavirus and *Photobacterium damselae*

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The need of high quality farmed fish production in conditions to be safe for the consumer and for the environment requires much efforts to maintain fish health and to avoid spreading of infectious diseases. This, in turn, requires much knowledge of immune responses against most dangerous pathogens that, in the case of Mediterranean sea bass (*Dicentrarchus labrax*) are a Nodavirus (retrovirus) (NNV) and the Gram-negative *Photobacterium damselae* subsp. *piscicida* (Phda). In a concerted scientific action aimed to study cellular and molecular responses of sea bass against these pathogens, fish (20-30 grams) have been challenged intraperitoneally with a modest dose of NNV that caused 12 % mortality (at 30 days), then treated again after 48 days with same virus. Alternatively, other fish groups have been treated with virulent Phda administered in water, followed by a similar treatment after 30 days. Fish from all experimental groups were sampled for molecular analysis by preparing RNA from homogenised organs and tissues, copying in cDNA and analysing by quantitative PCR for immunomodulatory expressed genes. This analysis was performed through PCR array using a protocol developed by our group. Fish were also sampled to obtain leucocytes for cellular analysis to investigate proliferation, lymphocytes profile, and sera analysed for the presence of pathogen-specific antibody by ELISA.

Proliferation of leucocytes in response to inactivated NNV was detected *in vitro* at day 43 in PBL and gills, and at day 73 in PBL, gills, and head kidney. No detectable *in vitro* proliferation was observed when adding Phda.

Serum analysis by indirect ELISA of pathogen-specific antibody showed that the treatment with NNV induced a specific response observed from day 47 onwards. ELISA analysis of Phda-treated fish showed the presence of specific antibody at days 10 and 44. However, the observed presence of detectable amounts of antibody against NNV and Phda in control fish raises interesting questions.

Several genes cloned in sea bass have been used for expression array analysis using PCR methodologies, and results obtained with fish challenged with NNV showed modulation of antiviral proteins interferon (type I) and Mx, of inflammation-related proteins IL-1, Cox-2, IL-10, and TGF-beta, whereas T cell genes TcR, CD4, and CD8 were not significantly affected. When analysing transcripts from fish challenged with Phda, proinflammatory peptides

IL-1 and Cox-2 showed a potent stimulation at 6 h after challenge, whereas T cell genes were only slightly upregulated after boosting. Interestingly, none of the immunomodulatory genes analysed was downregulated by NNV and Phda challenges.

Developmental expression of MHC class II in sea bass *Dicentrarchus labrax* (L)

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The major histocompatibility complex (MHC) class I and class II molecules play a pivotal role in vertebrate immune response to antigenic peptides. MHC class II genes have been identified in various teleost fish species, but no reports on their expression in sea bass development are still available.

Sea bass eggs, larvae from 2 to 92 days post-hatching (dph) and juveniles, were analysed for the occurrence of *MHCII-β* transcripts. These were first detected in 4 dph larvae by RT-PCRs, while Q-PCR revealed their significant increase until 92 dph ($p<0.001$). An early role of the MHCII-β molecules in the larval development is therefore suggested. In fact, $CD4^+$ T cells appear later in sea bass (51 dph), when the thymus is well developed. At this stage the i*n situ* hybridization of *MHCII-β* mRNA labelled cells in the inner zones of the thymic paired glands. From 75 dph on, the signal was detected in the thymic cortex and mainly in the outer and inner medulla. In one year old thymus numerous stromal cells were MHCII-β^+.

This report enlightens possible roles of stromal components in the mechanisms of thymocyte selection in fish.

Mx protein and interferon in sea bass (*Dicentrarchus labrax* L.): an evolutionary perspective

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The interferons (IFNs) are a large family of soluble cytokines involved in the immune response against viral pathogens. Three families of IFNs have been identified in mammals (type I, type II and type III) and, recently, homologues of type I and type II genes have been found in various teleost fish species. Our work has been focused on the identification of IFN and Mx homologues from sea bass (*Dicentrarchus labrax*), a fish of high economic impact for South Mediterranean countries. Moreover, we analysed the IFN gene structure and the expression of both IFN and Mx by real time PCR after stimulation with poly I:C. Finally, we predicted by template-based modelling the 3D structure of IFN and hypothesized its possible residues of interaction with the putative sea bass IFN receptor on the basis of the correspondent human complex. The sea bass IFN cDNA consists of 1047 bp that translates in one reading frame to give the entire molecule containing 185 amino acids. The analysis of the sequence revealed the presence of a putative 22 amino acid signal peptide, two cysteine residues and three potential N-glycosylation sites. Four Mx protein cDNAs were obtained and they translated in a putative protein of 652 amino acids. The sea bass IFN gene contains four introns as with other type I IFN teleost genes, except medaka that contains three introns. Real time PCR was performed after poly I:C stimulation of DLEC cell line and head kidney leukocytes to investigate the expression of sea bass IFN and Mx and an induction was observed for both genes. The predicted 3D structure of sea bass IFN is characterized by an “all-alpha” domain that shows an “up-down bundle” architecture made of six helices (ABB’CDE). The two cysteine residues present in the sequence (i.e. Cys^{23} and Cys^{126}) are in a position and at a distance that suggest the possible formation of a disulfide bridge that may stabilize the structure. These data add new insights on the evolution of the IFN system in teleosts and vertebrates more generally.

Identification of vaccine candidates against *Photobacterium damselae* subsp. *piscicida*

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Photobacterium damselae subsp. *piscicida* is the etiological agent of pasteurellosis, which is one of the most devastating bacterial diseases affecting the culture of gilt-head seabream and seabass in Mediterranean area. To date, efficient vaccines against the pathogen are not available. The aim of this study is the identification of vaccine candidates against *P. damselae* subsp. *piscicida*, using the reverse vaccinology, a new approach which starts from the in silico analysis of the genome sequence.

A genomic cosmid library of *P. damselae* subsp. *piscicida* strain NCIMB 2058 was constructed. Sequences, obtained by the shotgun method, were analyzed in silico by Glimmer 2.13 and Blastp for the identification of the proteins potentially encoded by the bacterium. Protein localization was predicted by Psortb, Tmpred and SignalP softwares, to select surface-exposed or secreted proteins. The analysis of 12 cosmid clones (421575 bp) identified 297 ORFs, 138 of which have a cellular localization spanning from the inner membrane to outside the bacterium. Among these proteins, 35 potential vaccine candidates were selected for expression as recombinant proteins. ORFs were cloned into pET-21b vector, expressed as C-terminal His6-tag proteins in *E. coli* and purified by metal-affinity chromatography. The potentiality of each antigen to become a vaccine ought to be tested by in vitro assay and by challenge experiments in fish. The complete genome sequencing of *P. damselae* subsp. *piscicida* will provide us with the inclusive set of

proteins potentially encoded by the bacterium and new antigenic molecules to use for vaccination.

Analysis of the Immunoglobulin heavy chain gene locus of the Antarctic teleost *Chionodraco hamatus*

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In teleosts, as in mammals, Immunoglobulin heavy chain (IgH) primary transcripts are alternatively spliced into mature transcripts encoding either the membrane receptor or the secreted antibody. However, the cryptic donor splice site in the last heavy chain constant domain (CH4), used in mammalian IgH gene, is absent in teleosts IgH. Thus, the exon for the transmembrane region splices to the 3' end of the CH3 exon. As a result, the synthesized protein lacks the entire CH4 domain. We have previously reported that in *Chionodraco hamatus*, as in the majority of Antarctic teleosts, an atypical splicing mechanism generates the membrane form, excluding two entire domains and including two additional 39-nt exons.

To investigate what makes this specific splicing type possible, a *C. hamatus* genomic DNA fragment was sequenced. This analysis revealed that the two 39-nt exons are part of reverse complement sequences (RCS) of an upstream gene region (CH3 exon). Three RCS regions are present in the gene locus (RCS1, RCS2 and RCS3). Each RCS shares, on average, 89.7 % of nucleotide identity with the respective counterpart that is present in the CH3 exon. Although sharing all the splicing signatures, the 39-nt sequence, present in RCS1, was missing in the mature transcript.

To explain the reason why the RCS1 39–nt element is spliced out from the transcript, the folding of the partial primary transcript encompassing the sequenced genomic region was predicted by using the mfold computational tool. The results showed a very compact structure stabilized by a long duplex comprising the entire CH2 and CH4 exons bound to the RCS1 sequence. The presence of both CH3 exon and the RC1 39-nt element in the duplex may account for the splicing mechanism observed.

C. hamatus belongs to the radiation occurred during the most relevant cooling geological period. This may suggest that the environmental changes directed the adaptive evolution of immunoglobulin gene locus.

An Immunoglobulin transmembrane dimerization motif conserved throughout vertebrate evolution

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Immunoglobulins (Ig), the key molecules of the adaptive immune system, are present in all vertebrate classes, but sharing very low sequence identity and differing in many features such as heavy and light chain isotype number, polymeric assembly, heavy chain domains number, carbohydrate content. However Ig molecules from different species do share a few important molecular characteristics. These include diversity of the variable domains achieved by somatic recombinatorial events, dimerization of the heavy chain, alternative splicing of pre-mRNA to synthesize the secretory or the membrane-bound Ig form. The functionality of the membrane-bound Ig depends on its ability to link the extracellular antigen recognition event to the cytoplasmic signal transducing machinery. In the present work we are aimed at identifying the structural motif that is universally conserved in vertebrate Igs, and is responsible for this activity.

Structural models of the IgTM homodimer were obtained using Ig from species of different classes: *Heterodontus francisci*, *Chionodraco hamatus*, *Pleurodeles waltl*, *Anas platyrhinchos*, and *Homo sapiens*. Several Molecular Dynamic simulations were performed in a lipid bilayer using two copies of models of Ig transmembrane (IgTM) sequences from each species, obtained by homology modeling. All predicted structures of the IgTM homodimers displayed similar packing interfaces, characterized by a high degree of surface complementarity. From the analysis of the models, we identified FXXXF as the motif presumptively responsible for the interaction and, in consequence, for the receptor stability and functionality.