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Report of Meeting

VIIIth scientific meeting of the Italian Association for Developmental and Comparative Immunology (IADCI), 1 and 2 March 2007, Area della Ricerca, CNR, Naples, Italy

Organizers: **U Oreste, MR Coscia**

Institute of Protein Biochemistry, CNR, Naples, Italy

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

Variomics of novel immunoglobulin-like transcripts in teleost fish

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The immune system uses a variety of rearranging and non-rearranging receptors to distinguish between self and non-self antigens. The rearranging receptors of the adaptive immune system such as the immunoglobulin proteins and T cell receptors have been studied in great detail in fish. It is hypothesized that the rearranging receptors, which form a subset of the immunoglobulin super family, arose from innate immune precursors that are not associated with somatic reorganization. Non-rearranging receptors of the innate immune system, which recognise largely unknown ligands, collectively denoted as pathogen-associated molecular patterns, have only recently been described in fish. Examples of these receptors are the Toll-like receptors and novel immune-type receptors (NITRs). Recently, highly polymorphic Novel Immunoglobulin-Like Transcripts (NILTs) have been described in common carp, which encode leukocyte receptors composed of a single extra-cellular immunoglobulin domain, a stalk, transmembrane and cytoplasmic region. Two receptors were isolated with opposing signal ability as indicated by the presence of either an immunoreceptor tyrosine-based activating (ITAM) or inhibitory (ITIM) motif.

Recently, we have extended these studies to salmonid fish. In Atlantic salmon an EST was retrieved with similarity to carp NILTs. The complete Sasa-NILT sequence was obtained by 3' and 5' RACE and was shown to encode the activating NILT receptor. We have analysed the presence of Sasa-NILT receptors in two unrelated Atlantic salmon individuals. This showed the presence of three framework Sasa-NILT sequences that were present in both individuals. In addition to these framework sequences, each individual has its own set of polymorphic Sasa-NILT receptors. The mechanisms that generate this receptor diversity are at present unknown. However, the haplotypic and allelic variation is reminiscent of the massive expansion and diversification of immunoglobulin-like loci encoded in the Leukocyte Receptor Complex in chicken.

Self/Nonself discrimination in ciliated Protozoa: the molecular basis

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As also a recent review in Cell on the "Quality Control in Self/Nonself Discrimination" points out (Boehm T. Cell 125: 845-858, 2006), comparative studies of the mechanisms that avoid self-mating in more ancient eukaryotes are thought to be of key relevance for shedding light on the control of specificity in self/nonself discrimination, as well as on the evolutionary emergence of the antigen receptors in the adaptive immune system. These studies, however, traditionally drive most attention on the self-incompatibility of plants, self-sterility and allo-recognition of tunicates, mating types of fungi. Scarce or no reference at all is made to ciliates. Nevertheless, the ciliate highly multiple mating-type systems are providing insightful information not only on the molecular basis of self/nonself recognition in more ancient organisms, but also on the central question of how new receptor/ligand pairs are generated in complex recognition systems.

This information essentially derives from: (i) NMR and crystallographic analyses (mostly carried

out in collaboration with the Kurt Wuthrich's laboratory at the ETH in Zurich) of the three-dimensional structures of a set of water-born protein signals (pheromones) produced by *Euplotes* species (Luporini *et al*. Curr. Pharm. Des. 12: 3015-3024, 2006), and (ii) the determination of the splicing mechanism by which the same cell controls its own specific diffusible signal and the (autocrine) binding receptor of this signal (Vallesi *et al*. Eukaryot. Cell 4: 1221-1227, 2005).

A novel approach for studying hematopoietic cells in *Hirudo medicinalis*

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Leeches have evolved a complex immune system that can recognize foreign antigens and can respond with a wide repertoire of reactions in relation to the non-self. Surgical wounds or cytokine injection induce the formation of an extensive network of new vessels and the proliferation of hematopoietic precursors. These cells exert their functional role migrating through the extracellular matrix towards the stimulated areas.

The different types of cells involved in leeches immune response have been characterized only *in vivo*, since its very difficult to isolate and culture these cells entrapped in the thick connective tissue. We establish an innovative system to isolate and culture specific population of leeches immune cells utilizing the injection of Matrigel Matrix gel added with different cytokines known to play a pivotal role in regulating proliferation, migration and differentiation of leech immune cells. After 48h from injection the Matrigel sponge is infiltrated by cells. The infiltrated Matrigel sponge dissected from leech is cultured in an appropriate medium. Two types of cultured cells have been obtained depending on the added cytokines. After one week cells cultured in Matrigel containing the Vascular Endothelial Growth Factor (VEGF) differentiated in endothelial cells CD34+ while those cultured in Matrigel containing the Monocyte Chemoattractant Protein-1 (MCP-1) differentiated in macrophages CD14+.

Our method provides a controllable protocol for repetitive isolation and culture of precursors cells from a "parenchimatous" animal and it is an excellent tool to select different types of cell population.

Aging and IL-6 immunoreactivity changes in the polychaete *Ophryotrocha labronica*

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The aging process is associated with dysregulation of the immune and inflammatory responses including modifications in the regulation and production of cytokines. IL-6 is a pleiotropic pro-inflammatory cytokine thought to play a role in age physiology, even if its possible modulation by aging mechanisms has not been fully defined. The morpho-functional modifications and IL-6 immunoreactivity during the aging process in a simple invertebrate model, the polychaete *Ophryotrocha labronica,* are reported. The comparison between newly-hatched, juveniles (at 6-11 setigerous segments, max 2 weeks), young adult females (at 14 setigerous segments, about 3 weeks) and 3 month old females showed significant structural differences in the nervous and genital systems. A reduction in the nerve area with a substantial depletion in neurons of the central system was found. A decline in oocyte growth and maturation was observed at the gonad level, even if sexually mature *O. labronica* continued to produce egg mass until week 16 of their lives. The age induced morphological modifications were associated to a different distribution of IL6-like molecules, that were detected in the central nervous system. A decreased number of reactive nerve cells and in particular in the anterior region of the brain of aged *O. labronica* was observed.

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

Evolution of helical cytokines: a structural approach

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Cytokines are small soluble factors retrieved in mammals and involved in several processes such as immunity and development. They are typically characterized by pleiotropicity, functional and receptor redundancy. In consideration of functional parallels between mammalian and invertebrate immunity, a lot of experiments have been dedicated to the unravelling of cytokine network in both protostomian and deuterostomian invertebrates. The presence of cytokine-like molecules has been evidenced by several morphological and functional investigations in different taxa of invertebrates, leading to the hypothesis that cytokines are molecules of ancient origin, present in metazoans before the division of protostomian and deuterostomian phyletic lines. However, all the recent molecular biology advances indicate that no sequence similarity can be retrieved between the known vertebrate cytokines and the whole genome of invertebrate species. On these basis, functional convergence has been proposed between

vertebrate and invertebrate cytokines. The functional convergence would be due to the lectin-like activity of vertebrate cytokines that can be retrieved also in some invertebrate molecules.

In order to unravel this unsolved matter, we have adopted a new bioinformatics approach able to isolate proteins whose structure is comparable to that of mammalian helical cytokines from EST and protein databases. Through this method we have isolated a molecule from *Drosophila melanogaster* databases that presents the structural characteristics of a helical cytokine (*Drosophila* helical factor, DHF). Functional experiments performed on third instars larvae and SL2 embryonic hemocyte cell line of *D. melanogaster* demonstrated that DHF expression was increased after different immune challenges.

From the present findings, it emerges that the contradiction between the amount of morphological and functional evidences and the absence of any homology between mammalian and invertebrate cytokines, could be explained by evolution of cytokine genes thereby conserving specific protein structures rather than amino acid or nucleotide sequences.

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Haemocytes of the cockle *Cerastoderma glaucum*: cell types and involvement in immune responses

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For the first time, morpho-functional characterisation of haemocytes from the cockle *Cerastoderma glaucum* was performed to identify circulating cell types and to study their involvement in immune responses. Haemocyte mean number was 5.5 (x105) cells/ml haemolymph (n=10). Two main haemocyte types were found in haemolymph: granulocytes (85 %), about 10 µm in diameter and with evident cytoplasmic granules, and hyalinocytes (15%), 8 to 14 µm in diameter, with a few or no granules. Most of the cytoplasmic granules stained *in vivo* with Neutral Red, indicating that they were lysosomes. On the basis of haemocyte staining properties, granulocytes and hyalinocytes were further classified as basophils and acidophils. Acidophil hyalinocytes were the largest haemocyte type (about 14 µm in diameter) and had an eccentric nucleus and a large cytoplasmic vacuole. Both granulocytes and hyalinocytes (except acidophils) were able to phagocytise yeast cells, although the basal phagocytic index was very low (about 2 %). It increased significantly (up to 26 %) after pre-incubation of yeast in cell-free haemolymph, suggesting that haemolymph has opsonising properties. Haemocytes also produced superoxide anion. Moreover, both granulocytes and hyalinocytes (except acidophils) were positive to some important hydrolytic and oxidative enzymes, such as acid phosphatase, non-specific esterase, acid esterase, and peroxidase. Lysozyme-like activity was recorded in both cell-free haemolymph and haemocyte lysate, although enzyme activity in cell lysate was significantly higher. Results indicate that haemocytes from *C. glaucum* are effective cells in immune responses.

Evidence for Stem Cell Factor-induced proliferation/differentiation in bivalve hemocytes

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Bivalve hemocytes comprise both granular and agranular circulating cells that are capable of non-self recognition through lectins and chemotaxis, and, most of all, phagocytosis. As with vertebrate phagocytes, bivalve phagocytes are equipped with both oxidative and non-oxidative killing systems related to activities of lysosomal enzymes. In bivalves, the process of hematopoiesis is still unknown. The most generally accepted belief is that hemocytes may originate from connective tissue cells, although hemocyte proliferation at sites of inflammation has been demonstrated. Stem Cell Factor (SCF) is a member of hematopoietic cytokines, a group of glycoproteins that regulate the growth and differentiation of hematopoietic progenitor cells and functionally activate mature neutrophils or macrophages. In this work the possible effects of recombinant human SCF on the hemocytes of the marine bivalve *Mytilus* sp. were investigated. The *in vitro* effects of SCF (50 ng/ml) on hemocyte functional parameters (lysosomal membrane stability-LMS and lysozyme release-LR) were first evaluated. SCF induced significant increase in LMS and decrease in LR, this indicating a reduction in lysosomal membrane fusion processes. Moreover, flow cytometry analysis showed that SCF significantly affected both hemocyte number and cell cycle; in particular, increases in the number of both granular and agranular hemocytes were observed. The results obtained with heterologous SFC support the hypothesis that common pathways involved in modulating activity, differentiation and proliferation

of immune cells are shared by invertebrates and vertebrates.

Characterization of hemocytes from *Polistes dominulus* (Insecta, Hymenoptera), target of the strepsipteran endoparasite *Xenos vesparum*

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Xenos vesparum (Insecta, Strepsiptera) is a macro-parasite of *Polistes dominulus*, a primitively eusocial paper wasp. SEM and TEM observations after artificial infections allowed us to follow step by step the parasite development inside the hemocoel of the wasp. The host-seeking stage is the triungulin (free-living 1st instar larva) which is able to "softly" overcome the structural barriers of the larval wasp (cuticle and epidermis) without any traumatic reaction at the entry site. The parasite molts 48h later to a 2nd instar larva, which moves away from the 1st instar exuvium, molts twice more without ecdysis and pupates, if male, or develops into a neotenic female. Some features result unusual: the encapsulation reaction involves the 1st instar exuvium (not the living parasite) and it starts only 48 h after host invasion; in addiction, no signs of melanization are visible. We suspect that *X. vesparum* inhibits host defense reactions during the early events of the infection and then the parasite seems to operate an elusion of *P. dominulus* immunity starting from the 2nd larval instar. We characterized the hemocytes present in the hemolymph of *P. dominulus* 3rd and 4th instar larvae (the main targets of triungulins) through morphological observations at TEM, SEM, light and phase-contrast microscopy; moreover we performed adhesion and phagocytosis functional tests and immunocytochemistry essays to check the presence of POMC-derived peptides and NEP-like molecules. Apart from the prohemocyte, the stem cell from which other hemocytes originate, two "types" or functional states are discernable, both of them adhering on glass slides and phagocytizing fluorescent beads, one type provided with structured and/or amorphous granules, the other one devoid of them.

Effect of cadmium exposure on phagocytosis and plaque lysis activity of *Paracentrotus lividus* coelomocyte

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Phagocytosis and plaque lysis activity (PLA) of coelomocyte from *Paracentrotus lividus* were examined after exposure to cadmium chloride (CdCl2·H2O), a potentially toxic metal salt, widely used in industry. *P. lividus* specimens were exposed at different Cd concentration (100, 200, and 400 µg l-1) for 24 hours (sampled at 0, 6, 12 and 24 hrs) at 15°C, in tanks containing artificial sea water (ASW) or injected with ASW containing the metal at 50, 100 and 200 µg l-1 in to the coelomic cavity. The treatment without Cd did not affect phagocytosis and PLA, whereas treatment with Cd, significantly lowered. This effect was dose and time dependent, presumably dependent on the cytotoxic effect of cadmium on coelomocyte as indicated by neutral uptake assay.

Session 3. Chairman: L Abelli, University of Ferrara, Italy

Invertebrate lectins present cytokine properties

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The origin and evolution of the innate immunity, including cell-cell, cell-cytokines, cell-lectins, cell-matrix interactions, appear to be product of cells and genetic markers for self recognition that grow with molecules and mechanisms to identify and destroy non-self. Lectins are components of a well-conserved protein-carbohydrate recognition system, the activity of most of them resides in a carbohydrate-recognition domain (CRD). They present an ample repertoire and have been proposed to mediate cell-cell or cell-extracellular matrix interactions in developmental processes, cell adhesion, inflammation and metastasis. Several immunomodulatory functions have been reported, among them mitogenic properties, opsonic properties, complement pathway activation and several immune responses.

Cytokines are the major regulators of the host defence processes and are involved in responses to exogenous and endogenous insults, tissue repair and recovery of homeostasis. Many cytokines are bifunctional molecules having, beside a receptor-binding domain, a CRD. The expression of the biological activity relies on the association between both domains. Several reports have also shown that cytokine-CRD can interact with various pathogens and presents the recognition site that contributes to pathogen elimination via opsonization and/or leukocyte activation. Lectin-like activities of several cytokines, including IL-1, have been described. Our understanding of invertebrate cytokine-lectin

biological functions and evolution are lacking. In some studies, similarities at the physicochemical level of vertebrate cytokines and functional invertebrate analogues have also been described. Among experimental approaches to identify cytokine-like molecules, antibodies neutralizing the activity of mammalian cytokines, have been used to screen for cross-reactivity with invertebrate factors in hemolymph.

Tunicates are a key group in chordate phylogenesis. In ascidian species, lectins are responsible for the in vitro opsonization, modulate cell proliferation activity, phagocytosis and complement activation, stimulate proliferation of mouse thymocytes and L-929 fibroblasts. Recently we have shown that, in the serum from the LPS-challenged *Ciona intestinalis*, IL1-like inducible components cross-reacted with anti-humanrIL1a antibodies. *Ci*IL1 with hemagglutinating properties, appears to be involved in sugar specific opsonization of yeast in an in vitro phagocytosis assay. Therefore a putative structural model of the opsonin include both CRD and IL1 epitopes. We propose an evolutionary model in which multifunctional costituive/inducible lectins can express cytokine activity with CRD responsible for pleiotropy and redundance, evolutionary conserved to guaranty a basic recogniton mechanism. Several inflammatory factors have been hypothesized as responsible for this process including components known in mammal innate immunity (cytokines, complement components, collagens) and phenoloxidase activity. A putative evolutionary model in which lectins are represented as multifunctional inducible molecules (cytokine-like function) involved in adult defence mechanisms, could be hypothesized. The lectin CRD, conserved in the evolution to guaranty a basic recognition mechanism, could explain lectin pleiotropy and redundancy as already suggested for mammalian cytokines.

A novel rhamnose-binding lectin from the compound ascidian *Botryllus schlosseri*

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Animal lectins play a fundamental role in invertebrate immunity, as they are involved in the recognition of microbial molecular patterns which, in turn, triggers various effector responses, such as opsonisation, encapsulation, activation of the pro-PO activating system, phagocytosis.

In a previous study, we purified by affinity chromatography and partially characterised a soluble $Ca2^{+}$-independent lectin, with specificity for b-galactosides, from the blood of the colonial ascidian *Botryllus schlosseri*. The molecule can agglutinate rabbit erythrocytes, is secreted by haemocytes upon the recognition of foreign particles and behaves as an opsonins (Ballarin *et al.*, 1999, 2000).

Recently, we purified further this protein by RP-HPLC, obtaining 4 lightly different peaks, likely isoforms of the same molecule. The MWs estimated using mass spectrometry ranged between 10.7 and 11.1 Kda. The lectin was digested with trypsin and tryptic fragments were sequenced by mass spectrometry. Blast analysis of the main sequences obtained indicated a high degree of homology with rhamnose-binding proteins, a family of S-type lectins described in sea urchin and teleosts. The specificity for rhamnose (and the similar melibiose) was successively demonstrated in haemoagglutinating inhibition assays.

We prepared a full length cDNA library from *Botryllus* colonies from which we obtained three full sequences of transcripts which, after BLAST analysis, resulted highly homologous to known genes for rhamnose-binding lectins. Their putative aminoacid sequences contained our tryptic peptide sequences.

Serum lectins in fish innate immunity: molecular and functional aspects

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Fucose-binding lectins (FBL) are present in tissues and fluids from invertebrates and vertebrates. The lectin repertoires in teleost fish are highly diversified and recently has been described the structure of the fucose-binding agglutinin that revealed a novel lectin fold (the “F-type” eel (*Anguilla anguilla*) fold), which shared a unique fucose-binding sequence motif contained both in carbohydrate-binding proteins and unrelated proteins.

In this report, we describe serum FBL from sea bass *Dicentrarchus labrax* and sea bream *Sparus aurata.* These lectins were purified, characterized, cloned and sequenced. Studies on structural aspects, biological activity, tissue distribution as well as ontogenetic aspects were carried out. In addition, results on inflammatory response and opsonic activity against bacteria suggested that *D. labrax* FBL is involved in innate immunity. Finally a new galactose binding lectin with agglutinating activity against bacteria purified from *Dicentrarchus labrax* serum was also described and compared with FBL.

Indifferentiating cells in the blood of the colonial ascidian *Botryllus schlosseri*: a morpho-functional charaterisation

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Colonies of the ascidian *Botryllus schlosseri* undergo a periodic tissue renewal in the take-over stage of the colonial blastogenetic cycle, during which an extensive apoptosis occurs in the adult zooid tissues and the senescent cells are progressively removed by circulating phagocytes. The haemocytes which circulate in the common vascular system also die partly by apoptosis during this stage. These cells are replaced by new haemocytes, likely differentiating from stem cells. Up to now, haemopoiesis was observed only in solitary ascidians in which haematopoietic noduli were described in the branchial wall. Nothing is known on haemopoiesis in colonial species, in the blood circulation of which two cell types with the morphology of undifferentiated cells are recognizable: haemoblast and lymphocyte. We have studied the cytochemical and immunocytochemical properties of these haemocytes: results indicate the haemoblast as a pluripotent stem cell since it shows a basophilic nucleus labeled either with Hoechst 33342 for euchromatin or anti-Ki-67 and anti-PCNA antibodies specific markers of nuclear proteins involved in cell proliferation and its plasma membrane is labeled by anti-CD34 and anti-CD100 antibodies, specific for haemopoietic cells in vertebrates. Commercial antibodies for cytokine receptors, like interleukin 1 receptor I (IL-1RI) and stem cell factor receptor (SCF-R) label haemoblast plasma membrane, suggesting the presence of growth factor receptors. Both lymphocytes and haemoblasts during the colonial cycle show a significant increase in concentration during the blastogenetic replacement. However, mitosis figures were rarely observed in circulating haemocytes. *In vitro* assays of haemocyte exposure to colchicine showed the presence of mitosis figures, which significantly increase after exposure to bacteria indicating a proliferating capability in blood circulation mainly as an immune response as observed in other invertebrates like molluscs.

Effects of different carbon dioxide concentrations on the adaptive immune system of cultured sea bass (*Dicentrarchus labrax*)

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Low oxygen and high carbon dioxide concentrations could affect health and welfare of farmed fish. This study evaluated acute and chronic effects of different dissolved carbon dioxide concentration (2-45 CO_2 mg/l) on specific immune response of sea bass. Fish were vaccinated against *Vibrio anguillarum* before exposure to CO2 and after 45 days were analysed for: a) the percentage of T and B lymphocytes in the leukocyte fraction of blood and head kidney (by flow cytometry using specific mAbs DLT15 and DLIg3 for T and B cells, respectively), b) proliferation capability of lymphocytes exposed to *Vibrio*; c) the serum content of anti-*Vibrio* Ig by captured-ELISA method and d) the agglutinating capacity of serum against *Vibrio* bacteria.

T and B lymphocytes significantly decreased ($P<0.001$) in fish maintained for 45 days at the highest CO_2 concentration respect to controls. The proliferation capability of head kidney lymphocytes was also significantly reduced in CO_2 treated fish. Also anti-*Vibrio* Ig content decreased (50 %; $P>0.001$) in CO2 exposed fish. Non-immunised showed a lower *Vibrio*-agglutination capability.
These findings evidenced the strong effect of CO_2 on circulating lymphocytes and in their specific immune function and the sensitivity of farmed sea bass to carbon dioxide concentration higher than 40 mg/l.

Thymic morpho-functional changes after hypophysectomy and bursectomy in chicken embryos

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Experiments of hypophysectomy or bursectomy were performed in chicken embryos in order to give more information on the role of hypophysis and Fabricius' bursa in the thymus development.

Hypophysectomy was performed on chick embryos at 36-40 hr of incubation. The thymuses were collected on day 18 and tested for: 1) anti-thymostimulin (TS) immune reaction; 2) histoenzymatic activities (LDH, SDH, NADH, NADPH, alfa-GPDH, Ca^{2+}-ATP-ase). The total thymic size was reduced and anti-TS, SDH, ATP-ase yielded negative reactions in the medullary epithelial cells. When hypophysectomized embryos received on day 12 a hypophyseal allograft from 18 day-old donor embryos, the thymic compartments improved and anti-TS immune reaction and enzymatic activities were partially recovered. Bursectomy was performed at 68-72 hr of incubation. The thymuses were collected on day 17 and were tested for the PCNA (proliferating cell

nuclear antigen) and CD3, CD8 and CD4 markers. A significant reduction of PCNA-immunoreactive lymphocytes was observed in cortex (P<0,001) and a significant decrease of anti-CD3,-CD4,-CD8 lymphocytes was evidenced in medulla (P<0,01). These findings confirm, at one hand, the key role of the hypophysis in thymic ontogenic development and, on the other hand, that bursectomy interferes with a correct differentiation of thymocytes and that there is an interrelationship between thymus and bursa at least during embryonic life.

Session 4. Chairman: N Parrinello, University of Palermo, Italy

Evolution of the complement system: invertebrate animal models

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In the past decade, in the context of the renewed interest in innate immunity, the Complement System has been investigated in increasing depth. One successful approach to analyzing the Complement System has involved the study of its evolutionary origin. The search for complement components has been carried out in very divergent species, aided by the powerful tools provided by computational biology and the genome projects that are ongoing in many invertebrate species.

A surprising result of these endeavours has been the finding that both C3, the key molecule of the Complement System, and factor B have a very ancient origin. In Cnidaria, these molecules seem to form a basic complement assembly that is able to opsonize bacteria through a primordial alternative pathway.

Carbohydrate-recognizing molecules, structural homologues of vertebrate ficolins, have been recruited by the Complement System more recently in the protostomian lineage.

High levels of complement complexity, comparable to those of mammals, have been reached in ascidians (Urochordata), which experienced many gene duplication events specific to the urochordate lineage. As a result of this gene expansion, ascidians exhibit a complex Complement System that operates through alternative and lectin pathways and exhibits pro-inflammatory and opsonic effector activities.

Expression pattern of C3 during *Ciona intestinalis* embryo development

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The identification of many complement components in ascidians indicates the presence of a complex Complement System, comparable to that of mammals, activated via an alternative and an MBL-mediated pathways. C3 activation-dependent pro-inflammatory and opsonic effector activities have been demonstrated in this subphylum. In particular, C3, the central molecule of the system, is expressed in *Ciona* blood granular amoebocytes and compartment cells, and the gene product is present in blood serum.

While the immune defense mechanisms and molecules of adult ascidians have received some attention, no information is available on the immune surveillance, if any, during embryo development.

To approach this topic, we have analyzed, in the present study, the spatial and temporal expression of C3 in *Ciona intestinalis* embryo. Following RT-PCR indications, we have carried out *in situ* hybridization experiments on developmental stages, from the unfertilized egg to the swimming larva. C3 shows an expression pattern restricted to mesenchyme cells and neural tissue cells. To extend these results Western Blot and immunochemical experiments have been also carried out.

Our preliminary data provide a first hint in defining the role of the C3 molecule, crucial in the innate immune response in the adults, during embryogenesis.

CiKLL, the ascidian multipurpose C-type lectin-like receptor

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C-type lectins, a family of diverse animal lectins characterized by a C-type lectin domain that serve for a broad range of biological processes, such as adhesion, endocytosis and pathogen recognition and neutralization, have been originally identified as carbohydrate-recognition molecules found in both invertebrates and vertebrates. The carbohydrate binding C-type lectin domains are part of a larger family of domains called C-type lectin-like domains (CTLDs) that seem to have originated by a process of divergent evolution from a common ancestor. Vertebrate CD94, one of the CTLD-containing molecules characterized by a lack of Ca^{2+}-binding sites, and therefore, a putative lack of sugar-binding activity, is one of several vertebrate natural killer lymphocyte receptors. CD94, forming heterodimers with NKG2 family molecules, regulates cytotoxic activity of NK cells towards target cells by interacting specifically with the MHC class I molecules, thus representing a *trait d'union* between innate and adaptive immunity.

In order to provide further information on the evolution of C-type lectins, we have carried out an in depth study on *CiKLL*, a homolog of the *BsCD94/NKR-P1*, a *CD94*-like gene identified in the colonial tunicate *Botryllus schlosseri*, present in *Ciona intestinalis* genome. CiKLL, showing intermediate structural features between a carbohydrate-binding protein and an NK cell receptor, is expressed in a blood cell type that is involved in the phagocytic activity during the immune response. Furthermore, CiKLL is expressed in the larva and during early metamorphosis in structures related to the nervous system. These observations are in line with the current speculations on gene cooption in the course of evolution particularly between genes involved in immunity and those related to developmental processes of the nervous system.

Preliminary characterization of a C1q-like transcript from the ascidian *Ciona intestinalis*

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C1q is a subcomponent of the C1 enzyme complex that triggers the activation of the classical pathway of the Complement System in the adaptive immune system of the mammalian species. C1q is known for its ability to bind antibodies as well as other ligands, including bacteria, viruses, parasites etc. C1q shows an hexameric assembly of three polypeptide chains, A, B and C chain. These chains possess the same topology, consisting in a collagen-like Gly/Pro-rich region, and a conserved C-terminal globular domain (C1q domain).

The analysis of the urochordate *Ciona intestinalis* genome has confirmed the absence of the pivotal genes for adaptive immunity in this species, which is phylogenetically at the basis of the vertebrate lineage. At the same time, this analysis has confirmed the presence of many complement genes, including a C1q domain-containing gene, *CiC1q*-like, encoding a protein with the same modular organization of the mammalian C1q chains.

To trace back to the invertebrates the origin of the classical activation pathway of the Complement System, we have undertaken the molecular and functional characterization of the CiC1q-like protein. We have verified the presence of CiC1q-like mRNA in *Ciona* blood cells, by PCR analysis: PCR products have been cloned and sequenced. The 3'-UTR sequence has been determined on clones obtained from the 3'-RACE procedure. The C1q domain of the CiC1q-like sequence has the 24-28 % of identity with the human orthologs. A phylogenetic tree generated by neighbour-joining method shows the relationship between the *Ciona* C1q-domain and the other C1q-domain orthologs. The ascidian C1q could either act as a lectin, like the lamprey C1q, or interact with other unknown membrane bound receptor/s, as in the case of murine SIGN-R1. Further investigations on CiC1q-like expression and biological function may help to shed light on the origin and evolution of the Complement System classical pathway and the C1q domain family.

Toll-like receptors in haemocytes of the colonial ascidian *Botryllus schlosseri*: preliminary results

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Toll-like receptors (TLRs) represent a well-known family of pattern recognition receptors, expressed by immunocytes, the importance of which in non-self recognition was demonstrated in both Vertebrates and invertebrates.

In the colonial ascidian *Botryllus schlosseri*, we used commercial anti-TLR2 and anti-TLR4 antibodies to inquire into the presence of Toll-like receptors (TLR) in haemocytes lysates. After SDS-PAGE, the immunoblot analysis revealed single protein bands recognised by the two antibodies, of 34 kDa and 32 kDa for TLR2 and TLR4, respectively.

Immunocytochemical investigation on monolayers of fixed haemocytes, previously exposed to *E. coli* LPS and yeast cells, revealed the expression of molecules recognised by TLR2 on activated phagocytes, whereas no labelling was observed with TLR4.

We also studied the role of NF-kB in the signal transduction pathway related to phagocytosis. Immunocytochemical analysis with anti-NF-kBp65 antibody revealed the labelling of the cytoplasm of untreated cells, whereas haemocytes exposed to yeast cells or *Bacillus clausii* spores showed a marked staining of the phagocyte nucleus. The NF-kB inhibitors Na-pyrrolidinedithiocarbamate and parthenolide, at sublethal concentrations, significantly inhibits both the ingestion of yeast cells by *Botryllus* phagocytes and the nuclear translocation of the activated factor. The same molecules have no effects on the morphology of haemocytes.

On the whole, our data suggest that, in our species, TLR are involved in phagocytosis and act through the activation of NF-kB.

Signal transduction in phagocytosis of the colonial ascidian *Botryllus schlosseri*: a preliminary approach

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In the course of our study on the role of immunocytes of the colonial ascidian *Botryllus schlosseri* in immune responses, we began to investigate the signal transduction pathways involved in yeast cell phagocytosis.

Both calphostin C, a specific inhibitor of protein kinase C (PKC), and H-89, a specific inhibitor of protein kinase A (PKA) significantly inhibit the increase in the phagocytic index. This indicates that both cyclic AMP, which activates PKA, and phospholipase C, which results in the production of IP3 and DAG (the former mobilising Ca^{2+} from intracellular stores, the latter activating PKC), are routinely required for phagocytosis.

In addition, manumycin A, inhibiting Ras activation, PD98059, inhibitor of ERK activation, SP600125, preventing JNK activation, SB202190, inhibiting p38 kinase, significantly inhibit yeast phagocytosis by *Botryllus* phagocytes. This suggest that the main MAP kinase pathways are involved in the ingestion of foreign cells.

The frequency of phagocytes expressing molecules recognised by anti-pan Ras antibody increase significantly when haemocytes were pre-incubated in the presence of foreign cells. Activated haemocytes also express molecules recognised by anti-p-ERK and anti-p38.

Therefore, a complex network of intersecting pathways is emerging and future research will aim to a better clarification of the main steps of signal transduction in ascidian phagocytosis.

Session 5. Chairman: MR Coscia, Institute of Protein Biochemistry, CNR, Naples, Italy

Teleost Immunoglobulins: genes and proteins

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The ability of teleosts to mount an antibody-mediated immune response has been reported for the first time in the last century, at the beginning of the forties. Serological methods, such as bacterial agglutination or hemolysis, provided the indirect evidence of the presence of antibodies in the teleost serum.

The introduction of protein chemistry and immunochemistry methods in the sixties allowed the purification and characterization of the immunoglobulin (Ig) molecules. The comparison with mammalian Ig showed evidence of many peculiar features of teleost Ig, such as isotype composition, polymeric assembly, lack of either secondary response or isotype switch.

Molecular biology tools have been introduced in fish immunology in 1989, when an *Ictalurus punctatus* Ig heavy chain (IgH) cDNA has been sequenced. Afterwards, nucleotide sequences encoding Ig genes have been obtained from more than 30 different teleost species. At present, data on Ig gene structure, regulation, and expression, are also available. The antibody repertoire, the *VH* gene segment diversity, the occurrence of different IgH and IgL isotypes, the alternative splicing of primary IgH transcripts have been deeply investigated in some model species.

The ongoing genome and EST sequencing of several teleost species, aided by computational biology, has enormously increased the knowledge of the immune gene organisation and functions: new IgH isotypes, new IgL gene segment organisation have been disclosed, allowing to draw a scheme of the vertebrate Ig evolution.

The knowledge on the most important molecule of vertebrate immunity has increased in the last decades at a very high rate thus prospecting fascinating results in the next future.

Molecular cloning, structural analysis and antigen-induced "*in vivo*" expression of interleukin-10 in sea bass (*Dicentrarchus labrax* L.)

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Interleukin-10 (IL-10) is a regulatory cytokine mainly involved in the suppression or deactivation of immune responses and is produced by macrophages and by the T-helper cells (subset Th2). Recently IL-10 has been discovered in the *Fugu* genome and cloned in different fish species.

Here we describe the homology cloning of this cytokine in the Mediterranean sea bass (*Dicentrarchus labrax* L.*)* and investigate its structure and *in vitro* and *in vivo* expression upon various stimulants. The full-length IL-10 cDNA consists of 1015 bp and is translated in one reading frame to give the entire IL-10 molecule containing 187 amino acids. A multiple alignment of the predicted translation of IL-10 sea bass molecule with other known IL-10 sequences showed the conservation of the fundamental features corresponding to IL-10 molecules. A comparative 3D modelling using human IL-10 as template showed that sea bass molecule is a symmetric homodimer, topologically similar to the structure of interferon-γ and about 70 % of the residues in each monomer assumes an α-helical conformation. Expression analysis by real-time PCR was studied at a basal level in the main lymphatic tissues and after *in vitro* stimulation with LPS and PHA in the head kidney. Moreover, an *in vivo* stimulation with the T-dependent antigen DNP human gamma

globulins (DNP-HGG), alone or in combination with an aluminium hydroxide emulsion, was performed.

Structural study of complex of MHC class I and co-receptor CD8 in sea bream by computational methods

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Comparative modelling represents the best predictive method for modelling the 3D structure of proteins. This method is applicable when the protein to be modelled is homologous to a protein whose 3D structure is known. The basis of this strategy is the observation that homologous proteins from different organisms can have low or high level of sequence identity, depending on the evolutive distance among them, but the 3D structure should be similar, being strongly related to the function played by that protein. On this basis, the 3D model of a protein can be created by similarity to the experimental model of known homologous protein.

In this work we have applied the comparative modelling strategy to model MHC class I and homodimer CD8aa sequences in sea bream and the related complex. The three-dimensional models of the sea bream molecules and complexes were created by using human and mouse template models. As the sequence identities between the sea bream proteins and the homologous template models were about to 30 %, we used an accurate procedure to search for the best alignment of sequences, in order to improve the quality of the modelling results. The obtained models present a global structure similar to the reference proteins.

Comparing the complexes obtained for sea bream and the mammals ones, we have evidenced some differences both in structural and in energetic terms.

Cloning, expression and structural analysis of the MHC class II β from sea bass *Dicentrarchus labrax*

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Major histocompatibility complex class II (MHCII) molecules present foreign peptides to T cells of the CD4 subset, and are thus fundamental components of the adaptive immune system. The MHCII proteins belong to the immunoglobulin gene family, bind to lysosomally generated peptides and are expressed only by B cells and antigen-presenting cells. They consist in a heterodimer (α and β chain) having two extracellular domains, a short hydrophobic transmembrane section and a hydrophilic cytoplasmic domain. MHCII genes exhibit an extraordinary degree of allelic polymorphism and are likely candidates as gene markers associated with disease resistance. Using degenerate primers corresponding to MHCII conserved regions of vertebrate sequences, we obtained an initial 190 bp product that, once sequenced and analysed by BlastX search, corresponded to a MHCII-β gene fragment. From this fragment we designed specific primers that were used in 3' and 5' RACE - PCR to complete the cDNA sequence. An alignment was performed using available MHCII-β aminoacid sequences and a phylogenetic tree was generated with the putative aminoacid sequences lacking the signal peptide. Moreover, two 3D MHCII sea bass models were obtained based on crystallographic mouse MHCII structures complexed with D10 T-cell antigen receptors and human CD4. Finally, specific primers were used to analyse by Real Time-PCR the MHCII-β expression in kidney macrophages stimulated with different concentration of sea bass rIL-1β and with LPS for 4h and 24 h, which are known to modulate MHCII expression.

Molecular models of *Chionodraco hamatus* IgM transmembrane region

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Membrane-bound immunoglobulin M (IgM) participates to the assembly of the B cell receptor (BCR). IgM consists of two μ heavy chains crossing the cell membrane and two light chains. The μ chain region traversing the lipid bilayer (TM region) is highly conserved among species and contains a universal motif for antigen receptors that is important for BCR assembly and function.

We analysed the TM region of Igμ from the Antarctic teleost *Chionodraco hamatus,* belonging to the Channichthyidae family. Its membrane μ chain is particularly interesting because generated by an unusual mRNA splicing mechanism.

We determined the complete nucleotide sequence of *C. hamatus* membrane μ. chain and analyzed the deduced amino acid sequence encoded by the TM exons. Using different computational methods, we predicted the length and

the polarity of the α-helical region crossing the cell membrane, and build a molecular model of the *C. hamatus* μ chain TM region, using the H helix of the photosynthetic reaction center of *Rhodobacter sphaeroides* as template. The stability of the model was investigated by molecular dynamics (MD) simulations. Models of a TM homodimer were also obtained by performing MD simulations using two copies of the helix, at a 14-16 Å distance between the centers of mass and in different orientations, as starting model. The obtained structures were related to the available experimental data collected on IgM TM region of different species.

Identification of a new *Trematomus bernacchii* immunoglobulin light chain isotype

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Immunoglobulin light chains (IgL) have been sequenced in 20 different teleost species. In each species one, two or three different isotypes have been described. We have previously identified in the Antarctic teleost *Trematomus bernacchii*, two immunoglobulin light chain (IgL) isotypes, referred to as *Trbe*L1 (distinguishable into two subgroups, *Trbe*L1A and *Trbe*L1B), and *Trbe*L3, based on comparison with the isotypes defined in other teleosts. In order to verify the presence of IgL isotype 2 in *T. bernacchii*, a PCR approach was chosen. Based on multiple alignment of IgL2 sequences from different teleost species, two oligonucleotide primers, complementary to the most conserved part of the FR2 region (sense) and to the 3' end of CL (antisense), were designed. The resulting PCR products were cloned into pGEM-T Easy vector and recombinant clones were isolated, sequenced, and found to belong to the *Trbe*L2 isotype.

Additional IgL cDNA clones were obtained by RT-PCR and 5' RACE using isotype-specific primers. The percentages of identity of the total 30 clones confirmed their distribution in three isotypes, one of them distinguishable into two subisotypes. By multiple alignment of the CL domains, conserved positions and isotype-specific residues were identified. To compare the molecular structure of each isotype specific CL domain, molecular models were built.

Multiple alignment and phylogenetic tree of the CL sequences from *T. bernacchii* and other teleosts indicated that each *T. bernacchii* isotype fell into one of the three teleost isotype groups.

Session 6. Chairman: L Mastrolia, University of Tuscia, Viterbo, Italy

Allelic polymorphism of the Igμ exons in the Antarctic teleost *Trematomus bernacchii*

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In mammals, the Immunoglobulin constant domains are relatively invariant, despite small amino acid differences are known to exist between products of the genes encoding IgA, IgM, and IgG among the population. Comparative studies in non-mammalian organisms have occasionally shown more than one Igμ constant domain sequence, but they were usually attributed to gene duplication.

To search for polymorphism in the Antarctic teleost *Trematomus bernacchii* Igμ gene, total RNA was extracted from the spleen of eight specimens caught in the same area. Two specific PCR primers were designed to amplify the entire constant region. Multiple alignment of the eight Igμ sequences, revealed that at least 51 positions were polymorphic. The individuals analyzed were found to be heterozygous or homozygous for each polymorphic position as expressing one or two variants. The highest number of polymorphic positions was observed in a particular region. In fact 30 out of 51 nucleotide substitutions were found to fall within the "hinge" region which connects the CH2 and CH3 domains. This region not only displayed extensive nucleotide variation, but also length diversity; in fact several sequences were one amino acid shorter as resulting from the usage of a different splice acceptor site as demonstrated by the analysis of the genomic DNA. Polymorphism was observed also at some potential N-glycosylation sites. The Ka/Ks ratios of the polymorphic positions showed typical values higher than one, indicative of positive selection acting to polymorphic residues to favor amino acid replacements and maintain allelic polymorphism.

Comparison with other non-Antarctic teleosts revealed that the high level of polymorphism in the "hinge" region is a peculiar feature of *T. bernacchii*. These results suggest that this property may have some biological significance, possibly related to modulating susceptibility/resistance to cleavage by bacterial or parasitic protease.

Secretory immunoglobulins in the skin of the Antarctic teleost *Trematomus bernacchii*

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The presence and localization of secretory immunoglobulins (Ig) in the skin of the Antarctic teleost *Trematomus bernacchii* has been investigated by using specific antisera in situ and in western blots. Analyses have indicated that L and H chains are present, that their molecular weights are similar to that of mucus Ig and that they are localised in filamentous, but not in mucous cells. Immuno-gold investigations have demonstrated that the Ig are dispersed in the cytoplasm and concentrated at the level of the endoplasmic reticulum thus suggesting a local production. In situ hybridizations confirm the hypothesis since demonstrate that filamentous cells synthesize mRNA for the Ig H chain. Hybridization also reveals that a significant synthesis of H chain mRNA occurs in mucous cells indicating a selective activation of post-transcriptional control mechanisms in the different cell types forming the skin in *Trematomus.*

B cells in lymphoid tissues of the Antarctic teleost *Trematomus bernacchii*

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Original data are presented on cytology and distribution of B cells in lymphoid tissues of the emerald rockcod (*Trematomus bernacchii*, Boulenger 1902), a bony fish living at sub-zero temperatures. B cells were identified by immunohistochemistry using antisera against homologous Ig H and L chains. Expression of membrane and secretory H chain transcripts and proteins was analised by RT-PCR and immunoblotting.

According to general features of the teleost immune system, B cells were numerous in the head kidney and spleen, while were fewer in the gills and intestine. On the other hand, a striking finding was the concentration of Ig+ cells in the thymus, clearly exceeding that found in all fish species studied so far. The differential distribution of membrane- and cytoplasmic-Ig+ cells (likely mature B cells, resting or stimulated) in the different vascular districts suggested a key role of head kidney and thymus in early B development, and of the spleen as the site of definitive maturation.

In the gills, Ig+ cells were scattered around the filament artery, in the pluristratified filament epithelium and around the outer marginal channel near the lamellar tip. In the intestine, Ig+ cells constituted a minor leucocyte population, localised almost exclusively in the lamina propria and submucosa. An increasing gradient (towards the stomach) in the number of Ig+ cells and eosinophilic granulocytes along the intestine suggested a higher inflammatory and immune responsiveness in the anterior segment, also mediated by hepatobiliary transport of immunoglobulins.

Localization of *CD8α* expressing T-cells in developing sea bass thymus: an hypothesis of positive selection

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These studies on thymus of the teleost *Dicentrarchus labrax* (L.) focused on differentiation and positive selection of T cells, crucial steps in the development of a functional immune system in all jawed vertebrates. Sea bass eggs, larvae (from day 2 until day 92 ph) and juveniles were analysed for developmental appearance of transcripts of two important genes in T cell function, *CD8α* and *TCRβ.*

RT-PCRs detected *TCRβ* transcripts in larvae from 25 days ph. Otherwise, *CD8α* expression was detected at day 51 ph when the thymus was well developed.

In situ hybridization of *CD8α* mRNA identified thymocytes in the outer and lateral zones of the thymic paired glands. From day 75 ph on, the signal was mainly detected in the cortex and the cortico-medullary junction. In one-year-old specimens *CD8α* and *TCRβ* expression patterns nearly overlapped, drawing a cortex-medulla demarcation in each thymic lobe. Large cords of *CD8α*+ TCRβ + cells lay in the medulla. A *CD8α*- TCRβ+ subcapsular zone was evident near the septa coming from the inner connective capsule that delimited the thymus. No signal was found in subcapsular and cortical epithelial cells.

The *TCRβ* and *CD8α* expression patterns demonstrated a compartmentalization of the thymus due to distinct localization of thymocytes at different developmental stages and suggested an hypothesis about positive selection in teleost thymus as described in mammals.

Localization of glucocorticoid receptor DIGR1 in tissues of teleost *Dicentrarchus labrax*

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Cortisol is a glucocorticoid that affects a wide variety of biological responses, including immune

functions. Virtually all tissues in the body are target organs and they can respond in different ways. The physiological response to cortisol is mediated through binding at two classes of intracellular receptors like mineral corticoids (MR) and glucocorticoids (GR) that act as ligand-dependant transcription factors. A cortisol receptor (DIGR1), (Vizzini *et al.*, 2007), from leukocytes of peritoneal cavity, previously cloned and sequenced, were employed for i*n situ* hybridization and immunohistochemical assays. The experiments were performed on brain, head kidney, spleen, gills, intestine and hearth. The mRNA and protein were expressed in the examined tissues. The wide expression and distribution of DIGR1 confirm the importance of the cortisol role in the maintenance of the homeostasis in the organisms.

Innate immune response of reared European sea bass *Dicentrarchus labrax* to different environmental and husbandry conditions

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Innate immune parameters were evaluated as indicators of health and welfare in reared sea bass *Dicentrarchus labrax* in relation to seasonal temperature changes, experimental carbon dioxide exposure (0-5, 15-20, 30-35 and 50-55 mg/l) for 45 days and different stocking densities (15, 30 and 45 kg/m^3) for 35 days.

Complement activity (ACH50) showed a seasonal trend, increasing during the summer and reaching maximum levels in September, with a positive correlation to high water temperature. Significant increase in serum lysozyme was measured in late summer and autumn, but any evident correlation with water temperature was found.

Hypercapnia induced a transient decrease in ACH50 activity within the first 24 hours of CO_2 exposure and in lysozyme levels in the first week of exposure. At the end of the experiment (45 days), there were no significant differences in lysozyme and ACH50 among hypercapnic groups and controls and compared to initial levels. The respiratory burst activity in hypercapnic groups was significantly reduced compared to controls after 45 days of CO_2 exposure.

High stocking density affected the ACH50 activity in sea bass with a significant decrease in fish maintained at 30 and 45 kg/m^3 for 35 days. Lysozyme activity did not change in fish reared up to 45 kg/m^3.

Results of present investigations indicate an influence of low water temperature, hypercapnia and high stocking density on innate immune response of sea bass that could be detrimental for health and welfare status.

Transferrins, nitric oxide and cytokines: complex responses to Marek's Disease Virus reinfection in a chicken lymphoblastoid cell line

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In previous works, we have shown that a relevant antiviral activity against Marek's Disease Virus (MDV) is exterted by ovotransferrin (Otrf), lactoferrin (Lf) (Giansanti *et al.* Biochem. Cell. Biol. 80: 125-130, 2002) and two ovotransferrin derived peptides (Giansanti *et al.* Biochem. Biophys. Res. Commun. 331: 69-73, 2005).

In addition, we have also evidenced the production of Otrf by a chicken lymphoblastoid cell line (MDCC-MSB1) induced by MDV, following reinfection with MDV (Giansanti *et al.* Biochem. Cell Biol. *in press*).

The aim of the present work is to verify if mechanisms other than tranferrins are involved in the defense against the above mentioned reinfection. For this reason, the possible role of NO, IL-8 and IFN-γ has been investigated. The data obtained indicate that NO production by MDCC-MSB1 is strongly enhanced in the presence of transferrins and after the reinfection. The NO production is completely inhibited by aminoguanidine (AG), an inhibitor of iNOS. It has also been observed that both cytokines stimulate the production of NO, and that the maximum stimulatory effect was obtained in the presence of IFN-γ plus Otrf or in the presence of IL-8 plus Lf. Also in this case, AG was able to completely inhibit the production of NO.