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WINTER SCHOOL

ON

Recent Advances in Mariculture Genetics and Biotechnology

4th to 24th November 2003

Course Manual

INDIAN COUNCIL OF AGRICULTURAL RESEARCH
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
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MOLECULAR BIOLOGICAL BASIS OF IMMUNE RESPONSES IN FISHES

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Introduction

The term immunity means exemption. This meaning was derived from exemption granted to certain categories of citizens in the case of compulsory military services. It has been recognized that those who recovered from epidemic diseases were exempt (immune) from further attacks. This knowledge helped Edward Jenner in 1798 to develop vaccine against small pox. Immune responses have its evolutionary beginning in animal kingdom some 400 million years ago. These responses have maintained a remarkable constancy at molecular and functional levels. The basic pattern of the protein molecules involved in this process has been maintained (conserved), while at the same time diversification of molecules to suit the emerging challenges was super imposed on the basic pattern. The immune phenomenon is believed to have evolved from the basic mechanism of self-recognition and non-self discriminations. Self-recognition and non-self discrimination appear very early in evolution and can be seen in marine organisms such as sponges. Aggregation of dispersed colonies of sponges is regulated by species-specific surface glycoproteins. Failure of adhesion of unrelated species amounts to a primitive form of graft rejection. The cells lining the cavity of sponges are able to capture microorganisms. Phagocytosis plays a role in the metamorphosis of insects in removing dead and disintegrated tissue.

Pollination and subsequent fertilization involves again recognition molecules. The same mechanism is involved in the fertilization of ova of marine fishes and other organisms by the spermatozoa of same species. Sea stars and corals are able to reject graft of unrelated forms. The self-non-self discrimination enables the organism to maintain specific association between its own millions of cells, while excluding changed self-constituents and potentially harmful parasitic organisms, such as protozoa, fungi and bacteria.

Basic pattern of Molecules

The cell surface molecules that are markers of self/non-self recognition are either carbohydrate or carbohydrate terminal groups on glycoproteins. The recognition factors themselves are proteins. Many of the marker proteins and recognition proteins have evolved either from β2 micro globulin or Thy-1 protein. The basic structures of immunoglobulin, histocompatibility antigen and phagocyte receptors indicate this. Each β2 micro globulin consist of polypeptide chain with110 amino acids of MW 12000 (Approximate). The vertebrates including fish have improved upon this primitive recognition mechanism and evolved a highly efficient system to deal with potential invasion from the co-existing biological world as well as aberrant or rogue cells evolved through spontaneous mutations (egs. Cancer). This system has retained all the primitive mechanisms such as, Phagocytosis,

agglutination of heterologus cells, lysis etc., while developing specific molecules and specific cellular mechanism against foreign molecules and cells. Hence, the immune system of vertebrates has two branches *viz.* innate immune system and acquired immune system; where as in invertebrates only the innate immune system exists.

The innate and acquired immunities are two branches of immune system, which are intimately interlinked. It is not possible to compartmentalize them into two separate units. Many times the help of one is required for the functioning of the other or one stimulates and modulate the other system. For example antibodies enhance the Phagocytosis of innate system. Antigen-antibody complex activates complement system. Interferons and interleukins stimulate and modulate acquired immune responses.

Innate immunity: non-specific defense mechanisms

The innate immune mechanisms are non-specific, since they are effective against a wide range of potentially infective agents. The main determinants of innate immunity are genetically controlled, varying widely with species, strain and to a lesser extent between individuals.

Surface barriers: 1. Mucus 2. Skin/ Exoskeleton. 3. Gills. 4. Gastro-intestinal tract.
Mucus: A layer of mucus (glycoproteins, proteoglycans and proteins) forms the interface between body and environment. Mucus entraps microorganism and mucus is continually replenished by mucus secreting cells, which inhibits the colonization of integument. The rate of secretion of mucus increases in response to infection or due to action of irritants. Lysozyme, bacteriolysin and complement cascade present in fish mucus are anti-microbial.

Skin: The skin surface of fish differs from that of higher vertebrates in that the epidermis composed of non-keratinized living cells. Epidermal integrity is vital to fish in maintaining osmotic balance and extending microorganisms. The epidermal healing response in fish is extraordinarily rapid, even at low temperatures. It involves a migration of malpighian cells from the periphery of wound surface rapidly closing the lesion, and is quite different from the scab formation, which occurs in mammals. Epidermis has resident migratory phagocytes. Malpighian cells are also capable of migration and Phagocytosis.

Exoskeleton: Crustaceans have exoskeleton made of chitin, which is frequently replaced.

Gills: Comprising such a large surface area of delicate epithelium, the gill is considered to be an important route of entry of microorganisms. The organ is protected by mucus production and a highly responsive epithelium resulting in hyperplasia, frequently seen in many infections. Pillar cells, that line brachial blood sinus are phagocytic.

Gastrointestinal tract: The lining of the tract is a mucous membrane, which secretes mucus in copious amounts. The digestive function of the gut provides an extremely hostile environment to pathogens. (1) Acidic pH in stomach (2) Action of digestive enzymes

[trypsin and pepsin]. In teleosts fish M cells and Peyer's patches are absent. However intraepithelial lymphocytes and macrophages together with eosinophilic granular cells situated in lamina propria are present.

Non-specific humoral factors: These include (1) growth inhibitors (2) inhibitors of enzymes or toxins produced by the pathogen (3) lysins (4) precipitins and (5) agglutins.
Growth inhibitors: These substances act either by depriving microorganism of essential nutrients or by interfering with their metabolism.

Metal ion binding proteins: These occur in the serum of all vertebrates including fish. Iron binding proteins (siderophilins) such as apotransferrins, ceruloplasmin and metallothionein inhibit the growth of bacteria. All these have been identified in fish. Apotransferrin binds two ferric ions. Ceruloplasmin oxidizes ferrous ions to ferric ions and metallothionein binds metal ions such as copper, zinc, cadmium and mercury. Metallothionein specifically binds to macrophage plasma membrane, initiating respiratory burst activity and signal transduction. Apotransferrins, which are also acute phase proteins, display anti -microbial properties by limiting the amount of endogenous iron available to pathogens including intracellular bacteria/ protozoan.

Acute phase proteins: Plasma proteins collectively termed as acute phase proteins increase in response to infections, and tissue injury. These include C-reactive proteins, serum amyloid A protein α_1 antitrypsin, α_2 macroglobulin, fibrinogen, ceruloplasmin, C_9 & factor B.

Cytokines: Cytokine related molecules are detected in fish and invertebrates. These are interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6 tumor necrosis factor (TNF), chemotactic protein-1 macrophage migration inhibition factor (MIF) and other peptide factors, which are involved in modulation of immune response and inflammatory reactions. Cytokines mobilize the host immune response, activate inflammatory reactions, and mediate bi-directional communication among various organ systems. Colony- stimulating factors (glycoproteins and peptides) regulate haematopoiesis and haematopoietic cell function, and transforming growth factors profoundly affect wound healing and cellular differentiation.

Interferons: are proteins that inhibit intracellular viral replication. The interferon has been reported from fresh water and marine fishes. They are classified into class I interferon (include α and β interferons) and class II interferons (γ interferons). With in each type there are several different forms. α interferons are produced mainly by lymphocytes and other nucleated cells. Interferon β is produced by fibroblasts and interferon γ is produced by T-lymphocytes and natural killer cells (NK or NC cells). The interferon producing cells when infected with virus (stimuli for INF synthesis are nucleic acids, bacterial cell walls, double stranded RNA and poly-synthetic nucleotides) synthesize and secrete interferons into extra cellular fluid. The interferons bind to specific receptors of uninfected cells. The antiviral effect is produced by derepression of two genes leading to the synthesis of two specific enzymes. One-enzyme catalyses the phosphorylation of ribosomal protein and initiation

factor elf-2, necessary for protein synthesis. This reduces the m-RNA translation in cells. The other enzyme catalyses the formation of short polymer of adenylytic acid, which activates a latent endonuclease, this in turn, degrades viral and host cell m-RNA. This establishes a cordon of uninfected cells around the site of viral infection restraining its spread. In addition to these effects it has several other immunological function such as major histocompatibility class II protein molecule (MHC II) expression on macrophages, increased Phagocytosis by neutrophils and macrophages. It also enhances activity of natural killer cells, T-lymphocytes, B-lymphocytes and other immune cells.

Eicosanoids: These are an important group of compounds derived from 20 carbon polyunsaturated fatty acids. In fish, eicoanoids are generally produced in organs rich in blood cells and in blood cells after ionophore stimulation. These eicosaoids include prostaglandins, thromboxans, lipoxins and leukotrines. They regulate blood clotting, MHC II expression, inflammation and Phagocytosis.

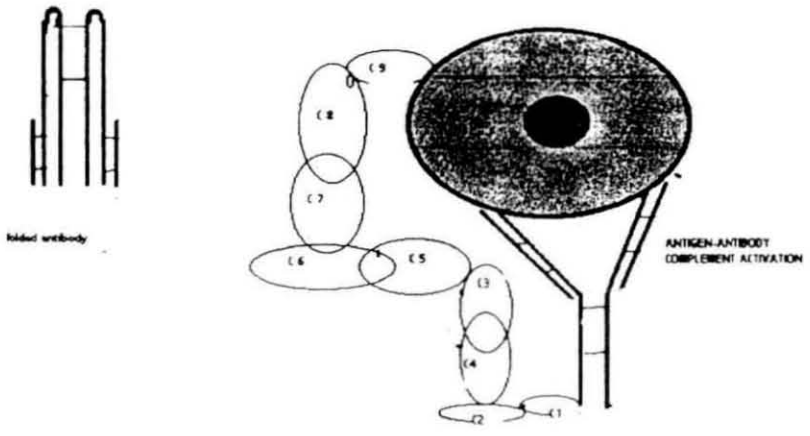
Enzyme inhibitors: Many pathogens produce enzymes in order to gain access to host body. Host tissue fluid and blood contain many factors, which neutralize these lytic enzymes. α_2-macro globulin inhibits a wide range of proteinases. α_2-macro globulin is able to entrap and form covalent linkages with proteins such as transforming growth factor (TGF) B_1, IL-1B and platelet derived growth factor BB. α_2-macro globulin thus regulates the action of coagulation cascades and complement cascade.

Lysins:

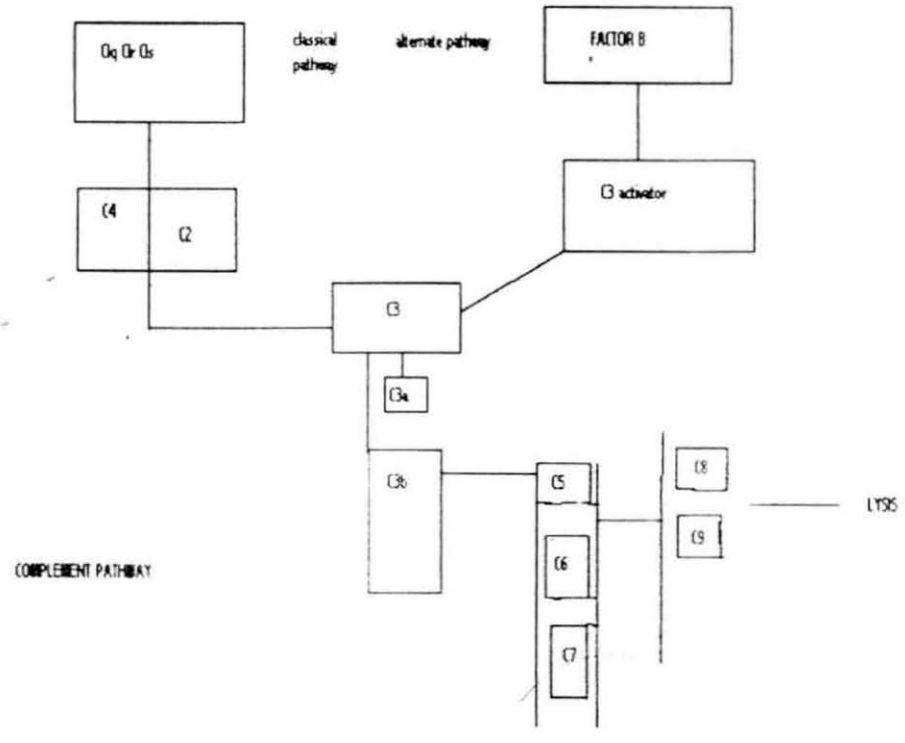
There are several enzyme systems, which cause lysis of heterogenous cells. These include complement cascade, pro-phenol oxidase, lysozyme and trypsin.

Complement: is an enzyme cascade system present in serum and tissue fluids, composed of twelve protein components. This is an extremely complex group of proteins. It has a broad spectrum of biological activity. Complement activation may be linked to humoral mediators of inflammation including the kinin and clotting systems

On the biochemical level many similarities exist between complement of fish and mammalian complement. Fish complement is highly heat labile and neutralized at 45°C. The complement can be activated by two major mechanisms. 1) The classical pathway in which the antigen-antibody complex exposes complement receptors on antibodies to which complement factors are adhered and enzyme cascade is triggered leading to formation of holes on target cells (antigen) and final lysis



2) Alternate pathway-the bacterial endotoxins, polysaccharides like zymosan and inulin, which activate properdin or factor B, which convert complement factor C_3 into C_{3b} and C_{3a} and thus the entire cascade is triggered into action.



In fish, complement is found in serum and mucus.

Pro-Phenol oxidase system: This system comprises an enzyme cascade leading to the activation of pro phenol oxidase and other compounds with related activities. Pro-phenol oxidase on activation by β-1, 3 glucans and the zymosan cleaves to phenol oxidase, which

catalyses oxidation of tyrosine to quinones. Quinones polymerize to form melanin deposits. Melanin deposition is a common immune response seen in invertebrates. Conversion of pro-phenol oxidase to phenol oxidase is done by a protease named phenol oxidase activating enzyme. This enzyme is kept in check by a protease inhibitor.

Lysozyme: This is a low molecular weight basic protein found in blood leukocytes of vertebrates, haemocytes of invertebrates, serum, haemolymph and mucus. It functions as a mucolytic enzyme, splitting sugars off the glycopeptides of the cell wall of many gram-positive bacteria, resulting in their lysis. Lysozyme also plays a role in the intracellular destruction of some gram-positive bacteria.

Trypsin: Trypsin and trypsin containing cells are found in epidermis, gills and intestine indicating local production and its secretion into mucus.

Precipitins and agglutinins

C-reactive proteins (CRP) and serum amyloid protein A (SA): These are plasma proteins and forms part of acute phase proteins. CRP binds to phosphoryl ester groups of bacterial cell wall, which contain phosphoryl choline. This binding is Ca^{++} dependent and activates complement. It has a structural analogy to limulin of horseshoe crab. CRP enhances the migration, Phagocytosis and respiratory burst of phagocytes. CRP can act as opsonin. CRP causes precipitation of heterologus proteins/ carbohydrates in non-immunized sera.

Serum amyloid protein A has got lectin binding property, by which they bind to bacterial cell wall glycoproteins

Agglutinins: These are a group of phylogenically conserved substances that are present in fish serum, mucus, bile and haemolymph of invertebrates. This group of substances includes lectins and other receptor specific substances. They act as opsonins and cause aggregation by binding to protein/ glycoproteins and/ or carbohydrate moieties that are free in solution or are constituent of microbes. Lectins are usually constitutive proteins or glycoproteins, which posses binding activity towards carbohydrate residues.

Cellular factors

Phagocytosis and the inflammatory response: various circulating and tissue fixed phagocytes rapidly engulfs any foreign particles, which enter animal body. In vertebrates they are the polymorphonuclear leukocytes and the macrophages; where as the haemocytes constitute the phagocytes of crustaceans and mollusks. The phagocytes contain digestive enzymes that degrade the ingested material. The phagocytes recognize, bind and ingest particulate materials. Recognition and binding take place through interaction of cell surface glycoproteins and cell wall carbohydrates of microorganism. More recently evolved mechanisms that utilize receptors in the phagocyte cell membrane for a part of the antibody molecule (Fc portion of antibody) and for a component of the complement (C3b).

Microorganism coated with antibody and complement thus adheres to the phagocyte and can then be ingested. Antibodies that enhance Phagocytosis are the opsonins.

Inflammation:

Inflammation is the dynamic process occurring in a viable tissue. It is the reaction of tissues to irritants/ disease causing agents. Inflammation begins following sub lethal injury to tissues and ends with the repair or healing of injured/ damaged tissue. . Following an injury the first sequence of change occur in local vascular system. These vascular changes are the result of release of pharmacodynamic amines from injured mast cells. The eosinophilic granular cells found in fish are believed to be the analogues to mast cells found in higher vertebrates. These cells are abundant in connective tissue of blood vessels as well as the stromal connective tissue, which form the structural framework of many organs and tissues. The bacterial products, physical and chemical trauma, products of damaged cells and complement factors released by immune response can produce injury to mast cells, which release the amines like histamine and serotonin. These amines induce increased blood flow and dilatation of capillaries. This increased blood flow through the area of injury result in redness (*rubor*). Dilatation of capillaries causes stretching of capillary fenestration, which allows colloids of the plasma to escape into the interstitial space. This results in increase in colloidal osmotic pressure, which attracts fluid contents of blood to tissue spaces, this may lead to swelling of the area (*tumor*) and the fibrinogen escaping with plasma proteins will be initiated to form fibrin mesh work. Dilatation of arterioles and pre capillary sphincters cause more capillaries to be opened. An increase in capillary and venule blood pressure is associated with dilation of vessels. Increased permeability of capillaries and venules leads to retardation of the flow and drop in blood pressure. The endothelial cells are activated to produce a lectin on their surface----selectin. The retardation of blood flow allows the heavy elements of blood to be distributed evenly. The leukocytes have on their surface the selectin receptor, which cause them to adhere to the endothelial layer. Then inside out signaling causes certain integrins on the leukocytes (β_1 and β_2 subunits) to gain affinity for molecules of immunoglobulin family; particularly those called **ICAM** (Inter Cellular Adhesion Molecules) on endothelial cells. These attachments help the leukocytes to stop, squeeze between endothelial cells and cross the blood vessel wall into the damaged or infected tissue.

Exudation of plasma/ serum

The changes in blood flow and the dilatation of capillaries and venules following enlargement of afferent arterioles lead to retardation of blood flow. This causes increased permeability and osmotic pressure change. Injury to cells leads to breakdown of macromolecules and they enter intercellular fluid resulting increased osmotic pressure. Loss of colloids into interstitial space through increased vascular permeability leads to fall in osmotic pressure of blood. Hydrostatic pressure at venules is increased due to vasodilatation of arterioles. Hence, there is increased accumulation of fluid at the tissue side; where as the

re-absorption of fluid from tissue is retarded due to fall in vascular osmotic pressure. The exudates formed will have plasma proteins including fibrinogen.

The exudates have the following functions. (1) Dilute the irritants. (2) Globulins are brought in contact with the irritants, which may neutralise the irritants (antibodies). (3) Fibrinogen in exudates forms fibrin scaffolding around the irritants, which will contain the spread of infection. The fibrin mesh will act as anchor for leukocytes to perform their functions.

Migration of leukocytes

Leukocytes emigrate to the tissues by amoeboid movement. The chemo taxis initiates this. Lipo-polysaccharides of bacterial cell wall released at the site are major chemo tactic agent. The cleavage products of complement, such as $\overline{C_{3a}}$, C_{5a}, C_{567}, lymphokines produced from stimulated lymphocytes and the products of granulocytes and monocytes all act as chemo tactic agents. Fatty acid derivatives derived from injured cell membranes such as leukotrines, 5 hydroxyeiosateraenoic acid (5HETE) are all chemo tactic.

White cells actively migrate through the fenestrae to enter the affected tissues. The cells penetrate junctions between endothelial cells and between basement membrane. They escape to the tissues at the points where basement membrane splits to accommodate pericyte. The collagenaese enzyme of leukocytes digests collagen. Lymphocytes are pinocytosed by endothelial cells and the vacuoles are exocytosed at the basement membrane region. Erythrocytes also leave through the fenstrae. The cells, which leave the blood, are 1) neutrophils, 2) monocytes, 3) eosinophils, 4) lymphocytes and 5) thrombocytes.

Neutrophils:

These are the first cells to migrate. They contain numerous cytoplasmic granules, which are lysosomes containing a number of enzymes capable of destroying the ingested organisms. They are hydrolytic enzymes, oxidative enzymes, proteolytic enzymes, phagocytin and lysozyme.

The proteolytic enzymes are two categories; acid proteases and neutral proteases. Acid proteases act with in phagosomes; where as neutral proteases degrade collagen, basement membrane, fibrin, elastin and cartilage. The neutral proteases are responsible for tissue destruction and this may release kinin and split complement factors C_3 and C_5 that in turn induces chemo taxis.

Opsonic serum factors coating on particles enable phagocytosis. They are complement fragments in fish. Immunoglobulin coated opsonisation is weak in fish. The phgocytosis by neutrophils release some quantity of enzymes to the tissues because the fish neutrophils are not efficient phagocytes. The phgocytosis and subsequent digestion are energy dependent. There are two types of digestion, oxygen dependent and oxygen

independent system. In oxygen dependent system there are two types. The superoxide system is characterized by increase in hexose monophosphate shunt activity (This is called respiratory burst, which can be demonstrated by Nitroblue tetrazolium staining-NBT). This generates superoxide anions O_3^-, H_2O_2, $^{\cdot}OH$ and $O^{\cdot-}$ These radicals affect macromolecules of the living organisms and thus kill organisms like bacteria. In the myeloperoxidase-peroxidase system myeloperoxidase enzyme increases the efficiency of H_2O_2 generating system by releasing halide ions (free halide). This system is more efficient in killing. In oxygen independent system---Hydrogen ions (H^+) reduce pH. Hydrolytic enzymes hydrolyses macromolecules; lysozyme split off sugars of bacterial cell walls. Fish neutrophils have very similar morphological and histochemical properties to mammalian neutrophils. They are present in kidney, spleen, blood and inflammatory lesions.

Monocytes

These cells appear in an inflammation in later stages. They are actively mobile and send numerous pseudopodia. The monocyte nucleus is ovoid, kidney shaped or indented. Nucleus is usually eccentrically placed. Nucleoplasm is condensed near its membrane. Cytoplasms are abundant and contain mitochondria, Golgi apparatus, rough and smooth endoplasmic reticulum. Once these cells reach tissues after leaving the blood stream they divide and mature. They are known as mononuclear macrophages. Their main function is phagocytosis. They can engulf large particles. They can recognise complement coated cells and particles through specific receptors that assist in phagocytosis. They also recognise molecules that have altered or denatured membranes and engulf them. They can secrete hydrolytic enzymes. Some of the macrophages mature into secretary cells with abundant cytoplasm and become closer to each other with indistinct boundaries. They are called the epithelioid cells. Differentiation to epithelioid cells occurs in chronic inflammation; where macrophages try to destroy irritants by secreting enzymes.

Mcrophages are wide spread in tissues but their concentration is more in reticulo-endothelial system. Reticulo-endothelial cells are found in interstitial tissue of kidney, spleen and endocardial lining of heart. Many macrophages in fish contain melanosomes within lysosomes. These are termed melano-macrophages. Melanin plays a role in bactericidal mechanism involving release of free radicals. Melano-macrophages form aggregates in parenchymal organs, which are called melanomacrophage centres.

Natural killer cells (Natural cytotoxic cells in fish)

Natural killer cells are large granular lymphocyte. They recognize structures on high molecular weight glycoproteins, which appear on the surface of infected cells. This recognition occurs through receptors on NK (NC) cells surface, which bring killer and target into close opposition. Activation of NK cells ensues and leads to release of granular contents into the space between two cells. The important factor perforin or cytolysin insert into membrane of the target cell and produce an annular pore. This induces cell death. The granules contain two serine estrases.

Eosinophils

Eosinophils are evolved to kill large parasites. A major basic protein MBP is located in the core of the granules. Cationic protein and peroxidase are present in the matrix of granules. Other enzymes are arylsulphatase B, phospholipase D and histaminase. It also contains dopaminase. Eosinophils have receptors for C3b. Activation produces respiratory burst and generation of oxygen metabolites. One of the granules can produce a trans-membrane plug like perforin. Most helminthes activate alternate pathway. The C3b allows eosinophils to adhere and activated eosinophils secrete MBP and cationic protein.

Haemocytes of in vertebrates have the same enzymes described for leukocytes and perform the phagocytic and degradation function seen in vertebrate cells.

Acquired immunity

Acquired immunity has two wings. (1) humoral immunity (2) cellular immunity or cell mediated immunity. Fish has developed both these systems.

Humoral immunity. The characteristic of this form of immunity is the appearance of globulins-immunoglobulins or antibodies in blood. These antibodies combine specifically with the antigen, which stimulate their production and lead to remarkable consequence.

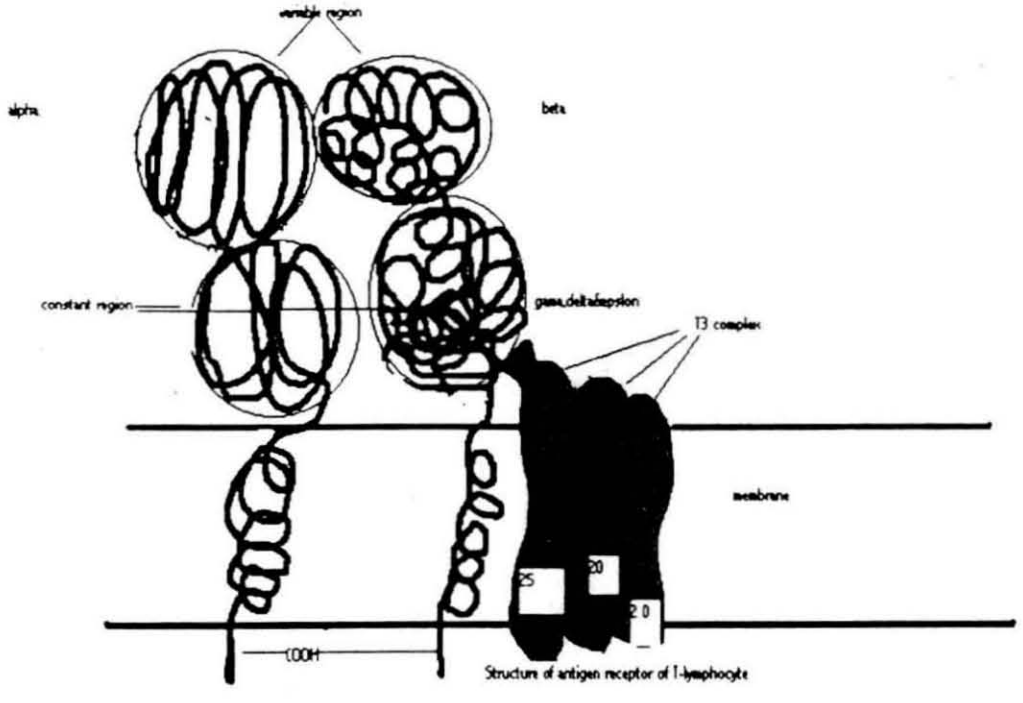
Cell mediated immunity (CMI).

Lymphoid cells may be induced, by prior exposure to antigen, to react subsequently directly with the inducing antigen and bring about cytotoxic effects, as for example on foreign cells from a graft. In both type of immunity the executors of the reaction are lymphocytes. Lymphocytes are fond in the circulation, the lymphoid organs and other tissues. In mammals and birds there are two distinct type of lymphocytes (1) originating or primed in thymus—T lymphocytes (2) bone marrow or bursa of Fabricius derived cells—B-lymphocytes. T lymphocytes are responsible for cell mediated immune responses (CMI), providing assistance for antibody production (helper function) and suppression of immune reaction.

B-lymphocytes are executors of humoral immunity. They transform into antibody or immunoglobulin secreting cells on antigenic stimulation. Subsets of T&B cells perform another function i.e. memory of any antigen encounter. In fishes dichotomy of immune system is also present but the details are not fully worked out. Evidence for T&B lymphocytes is available.

Lymphocyte subpopulations. The T lymphocytes have surface antigen receptors, which are α and β receptors in adult and δ γ receptors in embryonic stage. T lymphocyte receptor is a hetrodimer composed of α and β chain each of molecular weight 40-50 kD. Each chain

is folded into two domains, one having a relatively in constant structure, the other exhibiting far more variability. The variable region has the job of binding to antigen and MHC.



Both α and β chains are required for antigen specificity. In all immuno-competent T lymphocytes, the antigen receptor is non-covalently but still intimately linked in a complex with T_3, a molecule composed of three peptide chains (γ δ ε), which transduces the antigen recognition signal received by $\alpha\beta$ heterodimer to the inside of cell. The complete receptor is thus consisting of five peptides. In fish, thymocytes have a portion of Ig of heavy chain. In mammals no heavy chain receptor is found in thymocytes, where as they bear light chain related molecules.

T lymphocytes are differentiated in thymus. In higher vertebrates the priming of lymphocytes to T lymphocytes occurs. Thymus is protected against the ingress of foreign antigens by specialized endothelial lining of the blood vessels. The role of the thymus in fish is probably similar to that of mammals. The thymus consists of mainly lymphocytes in various stages of development and a few epithelial cells and macrophages. There is no differentiation into cortex and medulla. Thymus is a paired organ occupying dorsal region of brachial cavity and is extremely superficial, being situated with in the epithelium external to basement membrane. The blood vessels of thymus have specialized endothelium with tight junctions. In the embryo this is the first organ become lymphoid. A single layer of epithelium covers the thymus up to post fingerling stage. There are fenestration of 20mμ diameter in the epithelial layer. In older fish these fenestrations close and epithelium become thickened or thymus become deeply embedded in underlying tissue. Involution of thymus starts at sexual maturity, but it is a slow process in fish. Even in older fish some amount of thymic tissue will be discernable. Thymus is a primary non-executive lymphoid organ. Foreign particulate matter and protein antigen present in circulation are not able to

enter thymus lymphocytes also do not enter thymus. There is high mitotic activity in thymus and there is migration of thymocytes from thymus to spleen, anterior kidney and intestine. In adult fish thymus is responsible for regulating antibody response to T dependent antigen and suppressor activity. The developing thymocytes exhibit membrane antigen, which decide their future roles. These antigens are extensively studied in mouse. The mouse T cell antigens are termed Thy (τ) TL and Ly antigens. TL antigen is lost and the amount of Thy is reduced during differentiation. Ly antigen appear later in the development. Ly-1, 2,3 antigen expressing cells are the immature T-cells. Ly1, 2, 3-cell cell give rise to Ly-1 cells, Ly-2, 3 cells and Ly-1Qa$^+$ cells (60% Ly-1 cells are bearing Qa$^+$). Ly-1 cells are having helper function and Ly-1Qa$^+$ cells control the generation of suppressor cells. Ly-2, 3 cells have suppressor function on helper cells and B-cells. Ly-2, 3 cells also function as T-killer cells or cytotoxic cells. In other vertebrates the helper function is associated with CD4 receptor bearing cells and killer and suppressor function is associated with CD8 receptor. CD4, 8 cells are immature T-cells. Though clear-cut division of based on T cell antigen has not studied in fish thymus cells, the helper, killer, and suppressor functions are noticed in fish lymphocytes. Antigen difference between thymus lymphocytes and lymphocytes of anterior kidney are also evident. In mammals T lymphocyte antigen cross-reacts with brain tissue antigen. In fishes the lymphocytes, which are responsive to T-cell mitogens have antigens cross-reacting with brain tissue antigens.

B-cells have antigen receptors as single heavy chain of μ (Ig M). The B cell is produced in lymphoid tissues like anterior kidney and spleen. The B lymphocyte on binding with respective antigen through the receptor is stimulated to undergo multiplication and many of these cells acquire immunoglobulin secretary function.

Response to mitogens: Certain plant lectins are found to specifically stimulate division in lymphocytes. Phyto-haemagglutinin (PHA) and Concavalin-A (Con.A) are T-cell mitogen, where as lipopolysaccharides specifically stimulate B-lymphocytes. In fishes it is found lymphocytes cross-reacting with brain tissue antigen are responsive to PHA and Con.A, where as they are not responsive to lipopolysaccharides. Lipoplysaccharide responsive cells bear Ig M heavy chain molecule on their surface; where as PHA-Con.A responsive cells bear only a part of heavy chain Ig molecule.

The carrier hapten effect: One method to detect T-helper function is to estimate carrier hapten effect. Certain low molecular simple substances when injected into an animal will not produce an antibody response. If this low molecular substances is chemically linked to a large molecule and injected it will induce antibody response against it. The molecule, which is attached to the large molecule, is the hapten and the molecule, which is carrying it, is the carrier. Dinitro-phenyl (DNP) molecule will not induce immune response, where as; if it is linked to bovine serum albumin, it will elicit antibody response against DNP. In carrier hapten effect T-cell co-operation with B cell is required. Carrier hapten effect has been demonstrated in a number of fishes. It is also noticed that T lymphocytes of fish are capable forming rosettes with sheep erythrocytes.

Cell mediated immune reactions in fishes:

The markers of CMI are allograft versus host reaction. In graft versus host reaction an organism rejects organ/ tissue transplant from individual of the same species as well as from phylogenically different species. The rejection process will be faster, if the donor and recipient are genetically non-related. In this type of reaction no antibody is involved but only lymphocytes and macrophages. Once the animal reject a tissue transplant it will reject another transplant from the same donor at a short duration of time, thus rejection reaction induces immunological memory in recipient.

Scale transplantation and skin transplantation (both allograft and xenogrft) have been attempted in fish. In all cases rejection and immunological memory have been noticed. The lymphocytes of the recipients have been shown to retain sensitivity to donor antigens.
Delayed hypersensitivity reactions: These are lymphocyte-mediated reactions and lymphokine mediated reactions. These are specifically provoked by slowly evolving mixed cellular reactions involving lymphocyte and macrophages. The reaction is not brought about by circulating antibody but by sensitized lymphocytes. And can be transferred in experimental animals by means of such cells not by serum. The classical example is the tuberculin response. The animals or humans infected with *Mycobacterium tuberculosis*; 0.1 ml of 1 in 1000 dilution of protein extract of *Mycobacterium tuberculi* is given intradermally. An indurated inflammatory reaction in the skin appears about 24 hrs later and persists for weeks. The injection site is infiltrated with large number of lymphocytes and macrophages. Most of these cells are seen around small blood vessels. Among circulating lymphocytes there are a few sensitized lymphocytes, which on contact with antigen produce lymphokines and influence other lymphocytes and monocytes to aggregate at the site of antigen concentrations; and lymphocyte multiply at this site.

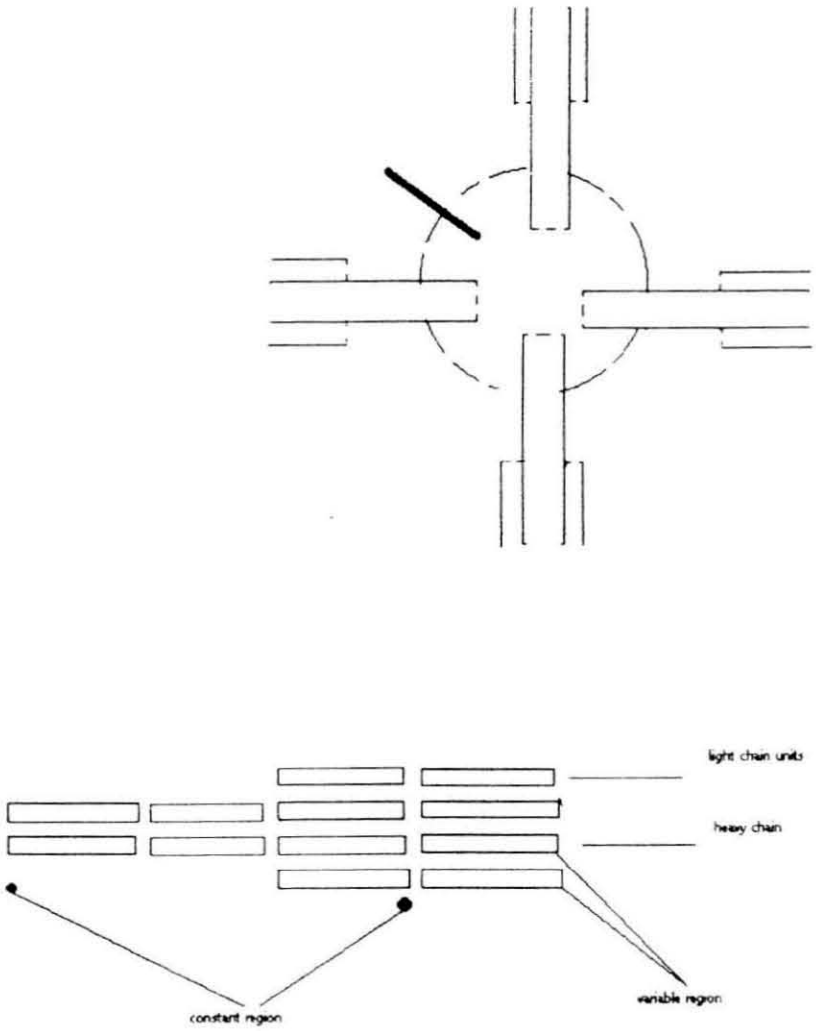
Delayed hypersensitivity can be demonstrated in fish by injecting T dependent antigens like BSA or tuberculin and making lymphocytes sensitized. Later an intradermal injection of the antigen will produce local inflammatory reaction like one described above. In chronic disease like bacterial kidney disease of trout delayed hypersensitivity has been demonstrated.

Thus it is clear fish has got a very good CMI response and the cells analogues to T lymphocytes of mammals are present in fish. The production of lymphokines in fishes can also be demonstrated by test like macrophage migration inhibition test and demonstration of chemotaxis in special chambers. Macrophage activation by the lymphokines can also be demonstrated. *In vitro* tests like specific contact cytotoxicity, mixed leukocyte reaction and antigen-induced blastogenesis of lymphocytes indicate fish has a strong CMI.

Humoral immunity

Presence of antibodies can be demonstrated in fish sera by agglutination, precipitation and complement fixation tests. In mammals and birds there are five classes (isotypes) based on the antigenic difference in the heavy chains. The basic structure of immunoglobulin consists of two heavy chains and two light chains. The heavy chain classes are μ,α,γ ε and δ (IgM IgA, IgG, IgE and IgD). The light chains are two types λ (lambda) κ (kappa). The basic structure is shown below. In mammals serum IgM is a pentamer, which consists five basic units linked in the form of a ring attached with protein called J segment. In teleost only IgM isotype is found. The serum IgM is tetrameric. Monomeric and dimeric form of IgM is also seen in mucus, bile skin and eggs.

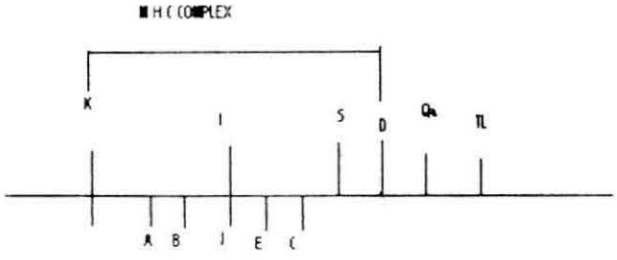
In mammals functional specialization is associated with heavy chain classes. In teleost antibodies can execute most of the reactions observed in mammals indicating heterogeneity at the level of function exist? It was also noticed with in species heavy chain molecular masses exhibited heterogeneity. There is also increasing evidence of local synthesis of secretary antibodies in bile, cutaneous and gastrointestinal mucus. Though immunological methods demonstrated only one type of antibodies in fish, there is structural difference with in the heavy chains. The differences may not have been amplified sufficient to produce antigenic differences in immunological reactions to classify them into different idiotypes.



BASIC STRUCTURE OF IMMUNOGLOBULIN-POSSIBLE EVOLUTION IN VERTEBRATES. THE MOLECULE IS 12 UNIT STRUCTURE. EACH CONSISTS OF OF POLYPEPTIDE CHAIN WITH 110 AMINO ACIDS OF MW 12000. EACH LIGHT CHAIN OF CONSISTS OFTWO BASIC UNIT AND HEAVY CHAIN HAS FOUR BASIC UNITS

Cell co-operation and major histocompatibility complex. The precise mechanism by which the immuno-competent cells co-operate involves the cell surface antigens. These antigens are glycoproteins and those involved in the rejection of grafts and these on transfer of a graft of tissue to unrelated recipients are recognized as foreign. The cell surface antigens are known as histocmpatibility antigen as the **major histocompatibility complex (MHC).** The genes controlling MHC are closely related to immune response genes and they are situated very close to immune response gene (Ir gene) loci in the same chromosome. The MHC genes have been studied in many mammals and rainbow trout. As a

model we take mouse. In mouse these genes are situated in chromosome number 17 and the graphic representation is given below.



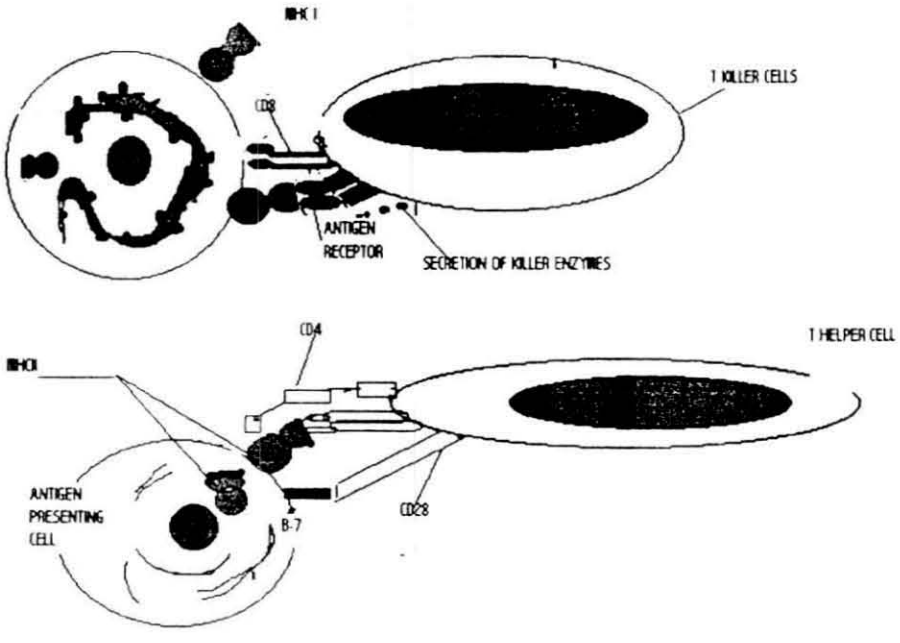
MHC COMPLEX	H2					Th	
MHC CLASS	1	2	3	1		1	1
GENETIC REGION	K	I	S	D		Qa	Th
GENES	K	ABJE	C4 SLP C2 Factor8	D	L	Qa-17	Th
GENE PRODUCTS	H2K	I-A, I-E, I-J	C4 C2 FB	H20 H-24		Qa 2-5	Th Qa1,6

The histocompatibility genes/ antigens belong to two classes. They are major histocompatability complex II and I. The genes coding for C4 and factor B, have made their way into the MHC region and are referred to as class III genes. Both class I and class II molecules are membrane bound heterodimers. Class I molecule consists of a heavy chain of 43 KD non-covalently linked to a smaller 11 KD peptide-β_2 micro globulin. The heavy chain has the globular domains α_1 α_2 and α_3, which protrude from cell surface. The hydrophobic section anchors the molecule into cell membrane and short hydrophilic end, which is C terminus, enters the cytoplasm.

The class II MHC is also trans-membrane glycoproteins having α and β polypeptide chains with molecular weight of 34 KD and 28 KD respectively. Both chains are folded to give two domains the ones nearest to membrane having considerable homology with β_2 micro-globulin and the characteristic Ig domain. It is seen that the I-J region code for more number of peptides than the space for genes it can hold, which that loci can hold. This includes suppressor and helper T lymphocyte receptors, immuno- globulins and other peptides. Probably the gene alleles mediate the selection of structurally related molecules indirectly, perhaps through idiopathic interactions involving T cell receptors. In the immunoglobulin system variability is achieved in each individual by a multigenic system. In MHC variability is achieved between individuals with highly polymorphic system based on

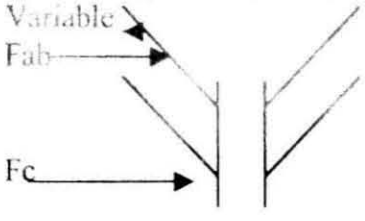
multiple alleles. It is very high in class I molecules. Multiple allelic forms can be generated by variety of mechanisms, point mutation, recombination, and homologous but unequal crossing over and gene conversion. This mechanism has been detected in mice. Most of the mutations contain clusters of multiple amino acid substitution and seem to arise by transfer of up to 95 nucleotides from class I Qa genes to$\alpha_1$and α_2 domains of H-2K. These findings have indicated that the large number of functionless Qa genes may represent a stockpile of genetic information for the generation of polymorphic diversity in the working class I molecules. Evidence for gene conversion has also been obtained for the class II genes.

All nucleated cells express class I molecules. These are abundant on lymphoid cells, less so on liver kidney and only sparsely on brain and striated muscles. Class II molecules are restricted to B-lymphocytes, macrophages and antigen presenting cells; however when activated by cytokines capillary endothelium and many epithelial cells express class II. MHC molecules have several physiological functions in addition to immunological functions. Here, we are more concerned immunological functions. It is known fact that detection non-self MHC activates the T-lymphocytes. The T-cell can recognize an antigen in association with MHC. The class I molecule is synthesized in the rough endoplasmic reticulum and transported to cell surface in a transport vesicle The MHC molecules have a groove which carries a native peptide (β_2). The T cells with CD8 receptors search the MHC I molecules. In viral infected cells the native peptide is replaced by viral encoded peptides or in cancer cells and old cells the peptide has different constitution. The detection of changed configuration in MHC leads to the multiplication and secretion of cytotoxic molecules by CD8 cells. Hence destruction of the cell occurs. The MHC class II also has a groove. The molecule synthesized in RER but held in cytosol. In B-cell the antigen captured by surface antibody receptor is delivered inside the cell. The antigen is broken down into small peptides; the class II molecule grabs the peptide and moves to surface (macrophage also degrades the antigens and fragments are exhibited on MHCII). The CD4 T- lymphocytes combine with the antigen presented on MHC II molecule. In an infection the B cells and macrophages express another molecule B-7. This combines with CD 28 on the T-cells. These bindings trigger T cells to secrete cytokines which initiate B-cell proliferation and antibody secretions. In the absence of non-production of B-7 molecules the T-cells become inactive or anergized.



Clonal Selection Theory: This theory proposes that the cells of the antibody-forming system have developed from random mutations resulting in the emergence of small numbers of cells or clones of cells differentiated so as to be capable of producing one or a very small number of specific antibodies. Contact by such differentiated cells with self or foreign antigens during fetal life before cells have reached maturity, would lead to suppression because the cells are annihilated by apoptosis.

After initial contact with antigen the cells of immune system retain memory, subsequent contact with antigen lead to quicker as well as amplified immune response. The immune system becomes more skilled with continued experience with the same antigen. The antibodies increase its combining capacity-avidity. This is due to the expansion of the clone. Memory involves the generation of long-lived T and B cells and changes in receptor involving generation of high affinity receptors bearing cells.



B-lymphocytes generate so many different antigen receptors. The antibody genes, particularly the variable region polypeptide genes are inherited as gene fragments. These fragments are joined together to form a complete gene in individual lymphocytes as they develop. The joining process it self generate more diversity. The enzymes that combine gene segments add random DNA bases to the ends of the process being joined as a result new genes are formed. Further diversity results from the assembly of protein chains into

complete receptor. Antibodies are made from two pairs of protein chains a heavy chain and light chain. The heavy chains are connected to form a **Y**, with the light chain located on the upper branches, along side the heavy chain. Each B-cell produces just one kind of light chain and one kind of heavy chain so that each B-cell makes unique receptor. The Genes for receptors of B-cells mutate extremely rapidly, when antigens activate B-cells.

Each heavy chain and light chain has variable (V) and constant (C) domain. The V domain is in the N terminal of both chains. V domain chain gene in heavy chain is formed by the recombination of four fragments-J, D and V. J for joining with C and D the diversity segment. The V region forms hyper variable region. V has 100 alleles in mouse, D 12 and J 4. These are randomly assembled in lymphocytes with non-coding introns in between. The introns spliced in m RNA during this splicing operation further diversity introduced by addition and deletion of nucleotides. The same mechanism is used in the case of light chains and generation of T-cell receptors.