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2001

Immunity and biological methods of disease prevention and control

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Almendras, J. M. E. (2001). Immunity and biological methods of disease prevention and control. In G. D. Lio-Po, C. R. Lavilla, & E. R. Cruz-Lacierda (Eds.), Health management in aquaculture (pp. 111-136). Tigbauan, Iloilo, Philippines: Aquaculture Department, Southeast Asian Fisheries Development Center.

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CHAPTER NINE

Immunity and biological methods of disease prevention and control

Jesus Manolo E. Almendras

Practically every multicellular organism, from invertebrates to vertebrates, is a potential host for various microbes. These microbes may spend some or all of their life cycle within, or upon, the bodies of their hosts. Any that gain entry to the tissues of their host may be rapidly distributed inside the host's body by its circulatory system. A host that does not have the means to protect itself from the entry and subsequent proliferation of microbes within its body can be rapidly overwhelmed. This chapter deals with the barriers that prevent microbial entry and the various internal defense mechanisms that are part of the host's arsenal in combating invading microbes. The latter part of this chapter discusses some of the biological methods of disease prevention and control.

The immune systems of fish and crustaceans have similarities and differences. Crustaceans are no different from other animals in that their defense system is largely based on the activities of the blood cells or hemocytes. These hemocytes, the crustacean equivalent of the vertebrate leucocytes, are capable of phagocytosis, encapsulation, nodule formation, and mediation of cytotoxicity. Another hallmark in crustacean immunity is the rapid sealing of wounds by blood coagulation to prevent loss of hemolymph and to immediately entrap invading microorganisms and arrest their dispersal in the body.

The fish immune system also has these non-specific mechanisms of defense but they can be differentiated from those of crustaceans as they have evolved an additional way of recognizing microbes. This recognition is the basis of what is commonly called the adaptive immune system or specific immunity. The adaptive system has two hallmarks that distinguish it from non-specific immunity. Firstly, recognition is performed by a receptor that exists in billions of different forms in an individual. This diversity endows the animal the ability to recognize any microorganism. Secondly, the adaptive system retains a memory of each particular microorganism to which it has been exposed. Memory allows the adaptive system to eliminate the same microorganism more effectively upon subsequent exposure.

The receptor that is responsible for these remarkable properties is the antigen receptor. These are found only in lymphocytes, which are found only in vertebrates. The substance or ligand, which the antigen receptor binds to, is called the antigen. Many substances that are foreign to the host can be an antigen, including proteins, polysaccharides and nucleic acids. These substances are usually components of the cell walls of microbes. There are two general catego-

ries of lymphocytes based on the antigen receptor they carry: T-lymphocytes and B-lymphocytes. The binding of antigens to their receptors triggers lymphocytes to become active in an immune response, a complex process usually referred to as lymphocyte activation. An activated B lymphocyte starts to manufacture large quantities of immunoglobulin molecules, which are then released into the blood. These soluble forms of immunoglobulin are commonly called antibodies.

THE FISH IMMUNE SYSTEM

Immunity in fish is mediated by two major systems: the innate or non-specific immune system and the adaptive or specific immune system. The innate immune system is thought to be of ancient origin and acts as the first line of defense against invading pathogens. It has no memory component and is active against a variety of microbial antigens. On the other hand, the adaptive immune system is present only in the vertebrates above the level of the agnathans. The most distinctive characteristics of the adaptive immune response are high specificity for microbial antigens and memory. Unlike the innate immune response, the adaptive response is not immediate since it would take time to synthesize specific antibodies against an invading antigen. Thus it constitutes the second but more specific line of defense. A summary of the components of the fish immune system is shown in Table 9 - 1.

Innate or Non-specific Immunity

Innate or non-specific immunity refers to various physical and cellular attributes that collectively represent the fish first lines of defense against infectious disease. These defense mechanisms come into play immediately or within hours of an antigen's or an invaders appearance in the body. The non-specific immune resistance includes the following levels:

Table 9 - 1. The major components of the immune response in fish

	Innate immune response	Adaptive immune response
Soluble Factors	Acute phase proteins Enzyme inhibitors Cell lytic enzymes Agglutinins	Antibodies
Cells	Phagocytes Natural killer cells	B – lymphocytes T – lymphocytes

Physical Barriers

The skin, including the scales in some fishes, and the mucous membranes of the digestive tract are physical barriers that protect the animal from harmful environmental agents and from invasion by microbes. Most of the fluids that are excreted or secreted onto epithelial surfaces, such as mucus and digestive juices, contain chemical components that are anti-microbial. Mucus is secreted by specialized goblet cells in the epidermis of fish and contains immunoglobulins (IgM). Mucus also contains precipitins, natural agglutinins, lysins, lysozyme, C-reactive protein and complement. However, the skin and other epithelial surfaces of fishes harbor a variety of microorganisms whose presence and numbers pose no threat to their health.

Soluble (Humoral) Barriers

The internal fluids (humors) of fish contain a number of substances that react with a variety of microbes to lyse, or coat them or to inhibit their growth. Among these substances are inhibitors of microbial growth (transferrin, lactoferrin, ceruloplasmin, metallothionein, cecropins, defensins, magainins), cell lytic enzymes (lysins, lysozyme, proteases), enzyme inhibitors, agglutinins and precipitins and interferons).

1. Acute Phase Proteins and Complement Factors

   The concentration of a number of protein types in the blood serum increases rapidly during an infection. These proteins are called *acute phase proteins.* One representative of the acute phase proteins is the *C-reactive protein* characterized by its ability to bind to the surface molecules of the cell wall of a wide variety of bacteria and fungi. When the C-reactive protein binds to the surface of bacteria, another group of proteins present in the blood, called *complement factors*, binds firmly with the immobilized bacteria and the whole complex of bacteria, C-reactive protein, and complement becomes more rapidly engulfed by phagocytes. This process of facilitating phagocytosis of bacteria by coating them with protein is called *opsonization*. In fish, C-reactive proteins are naturally present at a level that is 500 times higher than in mammals, a possible indication of the relative importance of the nonspecific resistance mechanism in fish.

   The complement factors of fish blood are also proteins. One of these proteins will react spontaneously with surface components of bacteria, notably the lipopolysaccharide (LPS) found on bacterial cell walls. The same complement factor also reacts with β-1,3 glucans which are structural components of bacterial and fungal cell walls. This reaction is called an activation because it elicits a sequence of reactions where one complement factor activates the next in a chain reaction that produces protein fragments with different properties. After activation, some of the complement factors are able to cause opsonization of bacteria, whereas others attain the ability to attract phagocytes.

   The activation of the complement system by external factors, such as LPS and β1,3 glucans is called the *alternative pathway* of activation. The complexes formed when antibodies react with antigens can also activate the

complement system. This is called the *classical pathway* of complement activation.

The different proteins in the complement system must act in combination in order to exert an antimicrobial effect. Moreover, lysozyme produced by phagocytic cells acts synergistically with the complement factors by its hydrolytic effect on the bacterial cell wall. Measurement of the level of complement factors and of lysozyme in the serum, and measurement of the phagocytic and bactericidal activity of phagocytes that operate in concert with these factors, provide ways to quantify the degree of non-specific resistance of an organism.

The C-reactive proteins, complement, and phagocytic cells constitute the most important elements of the non-specific immune system. Because this system exists with its same basic elements in all levels of the evolutionary system from marine invertebrates to warm-blooded animals, it is likely that it has its origin far back in the evolutionary process.

2. Transferrins and Lactoferrin

   Transferrin is a serum protein of 70 to 80 kDa molecular weight belonging to the group of acute phase proteins and present both in mammals and fish. Transferrin plays a possible role in delaying the start of microbial infections by binding the available iron and thereby depriving bacteria of an essential growth factor. The mucus secretions of mammals contain another closely related iron binding and antimicrobial protein called lactoferrin, but this has not yet been found in fish; lactoferrin does, however, enhance the disease resistance of trout when given in the feed.

3. Caeruloplasmins

   This is an acute phase protein present in fish that binds copper and other divalent metal ions. Caeruloplasmin acts as an oxidase that oxidizes divalent iron to ferric ions, which then bind to transferrin. By this reaction, ceruloplasmin contributes to depriving bacteria of available iron and divalent ions.

4. Metallothionein

   Metallothionein is a peptide rich in cysteine and with a high affinity for metal ions. Metallothionein has been found in several fish species. Its production is stimulated by metals in the environment and by endotoxins (LPS) from bacteria. It also deprives bacteria of essential metallic ions by sequestering them.

5. Enzyme Inhibitors

   Protease inhibitors that correspond to the mammalian α-2-macroglobulin are present in fish and are believed to play a role in retarding the invasion of pathogens or parasites. The other common groups of protease inhibitors are present in fish serum (inhibitors of serine proteinases, cysteine proteinases, aspastic proteases, metallo proteinases) and some of these belong to the acute phase reactants. Virulent pathogenic microorganisms secrete pro-

teolytic enzymes to digest and penetrate the tissues of their host. It is believed that the protease inhibitors of fish serum play a role in defense by neutralizing the proteolytic enzymes produced by these pathogens.

7. Cell Lytic Enzymes

   Lysozyme is a bacteriolytic enzyme occurring both in plants and animals. There are large variations in lysozyme activity in different fish species. Lysozyme is present in most tissues and secretions of fish, and the level seems to vary in relation to environmental conditions. Stress due to handling of fish and to pollutants reduces lysozyme activity in fish. Because lysozyme is produced in macrophages, the reduced level may be a reflection of a reduction of the macrophage function

   Fish serum contains molecules other than lysozyme that cause lysis of bacteria and probably have a function in the non-specific defense to infections. Fish mucus also contains a trypsin-like protease activity that is able to cause lysis of Gram-negative bacteria. This enzyme is produced in the mucus-secreting cells of fish and may function in concert with lysozyme and hemagglutinins in non-specific defense.

8. Agglutinins

   Fish serum, skin, and mucus contain factors that resemble immunoglobulins or antibodies in their ability to agglutinate and cause lysis of foreign cells and bacteria. These agglutinins are non-specific and they correspond to lectins of invertebrates in their ability to recognize and bind to single sugars on the bacterial surface and mediate phagocytic reactions

Cellular Barriers of the Innate Immune System

1. Inflammation

   Inflammation is the observable condition that accompanies damage to the body. It is a localized response to tissue injury and to invading microorganisms, characterized by infiltration by granulocytes and macrophages, removal of dead cells and foreign cell and debris, followed by tissue repair. Inflammation confers protection by walling off an infected area from the rest of the body. This type of response in fish has been reported against bacterial, viral, fungal, protozoal and parasitic infections. In the higher vertebrates especially in mammals, inflammation involves mast cell degranulation and the release of vasoactive substances. These cause vasodilation, which increase blood flow and vascular permeability, and adhesiveness of vascular endothelial cells for phagocytic blood cells.

2. Natural Cytotoxic Cells

   Some population of cells in fish display a non-induced and non-specific toxicity to foreign cells. The non-specific cytotoxic cells in fish are equivalent to the natural killer cells of mammals. They differ from their mammalian counterparts by being able to destroy a wider range of foreign cells, and they can even destroy multicellular parasites that attack fish.

3. Phagocytosis in Fish

In fish, macrophages, monocytes and granulocytes are phagocytic and in some species neutrophils are also phagocytic. Intracellular killing by teleost macrophages is similar but slower than found in mammals. These killing activities include lysosomal enzymes, alkaline and acid phosphatases and peroxidases. Activated macrophages produce oxygen metabolites such as super oxide anion in a process known as the respiratory burst. These oxygen radicals are bactericidal.

Adaptive or Specific Immunity

The concept of specific immunity includes three important components: the ability to recognize and respond selectively; the ability to recognize and respond preferentially to foreign substances and the ability to respond better on repeated exposure to them (memory).

Antigens

The specific substances that trigger an immune response are called antigens. Most antigenic substances are large molecules like proteins, polysaccharides and nucleic acids, but usually the immune system only recognizes and responds to a small part of these large molecules, called the antigenic determinant or hapten. So, each antigenic substance can have many different haptens in one large molecule. Small foreign molecules can also act as haptens when attached to a larger molecule.

Cells

The two types of cell that are involved in specific immune responses are lymphocytes (the B and T lymphocytes) and antigen-presenting cells (APCs), which include macrophages (Figure 9 -1).

Receptors

Lymphocytes, but not APCs, have membrane receptors for specific antigens. These receptors are proteins that specifically recognize and binds to an antigen. They are the means all lymphocytes use to recognize antigens and some use to respond to them (Figure 9 -1).

Humoral vs Cellular Immunity

There are two different forms of specific immunity: humoral (or antibody-mediated) and cellular (or cell-mediated). Cellular immunity acts by direct cell-to-cell contact to protect the body against viruses that have infected its own cells and against tumor cells. Humoral immunity acts by the secretion of soluble proteins (antibodies or immunoglobulins) that circulate in the blood and lymph where they can combine with antigens and neutralise them.

Humoral immunity

Active and Passive

Humoral (antibody-mediated) immunity can be either active or passive. Active immunity occurs when the antibodies are made within the body's immune

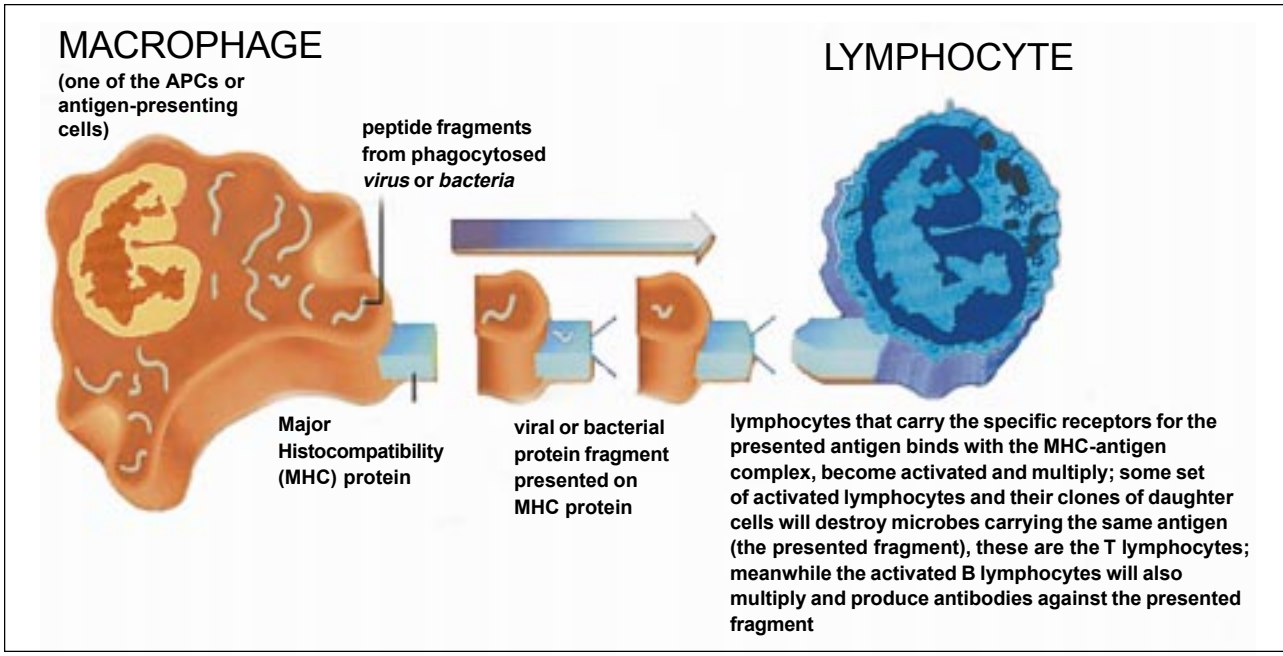

Figure 9-1. The process of presentation of a foreign peptide or protein fragment from an invading microbe by the MHC protein of a macrophage and the binding and activation of specific lymphocytes that carry the corresponding receptor to the presented fragment

system. It is passive when the antibodies come from outside the body. An example of this is the colostrum of mammalian milk, for a few months after birth a mammalian infant is naturally protected by passive immunity gained through antibodies secreted in its mother's milk, this is also true in some fish eggs and larvae that contain some antibodies produced by the parent fish. Antibodies can be extracted from plasma and injected into a patient as an artificial but temporary means of providing immediate protection against certain antigens.

Antibodies (Immunoglobulins - Ig)

Antibodies are Y-shaped proteins formed from two pairs of polypeptide chains (two heavy and two light chains) held together covalently by disulfide bonds. Each antibody can bind selectively to one type of antigen. But each arm of the Y has a binding site, so each antibody molecule can bind to two molecules of the same antigen. The stem of the Y can be recognized by other cells of the body, so it helps determine what happens to an antigen-antibody complex.

Antibody Diversity

The binding sites of an antibody are specific and highly selective for a single antigen. The body is capable of making an antibody that can combine with any conceivable antigen. The genetic mechanisms that allow this are complicated, but very fascinating. In addition, any particular antibody can occur as different classes of immunoglobulin (IgG, IgM, IgE, IgA, IgD), which are effective in different circumstances. They differ in the kind of protein that forms the stem of the Y (parts of the pair of heavy chains), although they have the same anti-

genic specificity. The cells (activated lymphocytes) that make a specific antibody can switch from making one class to making a different class, but always of the same antigen specificity.

Antibody Production

Plasma Cells

Plasma cells are the effector cells of humoral immunity. They synthesize and secrete antibodies (IgM and IgG). Plasma cells are B lymphocytes that have been activated by interaction with their matching antigen. The antigen receptors on the membranes of unstimulated B lymphocy'tes are IgM and IgD class antibodies that have the same specificity as the IgG that they will eventually produce as plasma cells. Plasma cells are mostly located in lymphoid organs (but not in the thymus) but are sometimes released into the blood.

The purpose of B lymphocyte stimulation is to form enough plasma cells to produce useable amounts of antibodies. It is a complex process. Besides the B lymphocytes themselves, it involves accessory cells (e.g. macrophages) and a specific class of T lymphocytes (helper cells). It also depends on the presence of special membrane proteins (major histo-compatibility or MHC proteins) on the surface of these cells and the release of different paracrine and autocrine chemical messengers (cytokines) by them.

There are two classes of MHC proteins, MHC-I and MHC-II. All cells (except RBC's) have MHC-I proteins. Only a few cell types have MHC-II proteins. These include macrophages and other accessory cells (including B lymphocytes) that can function as Antigen Presenting Cells (or APC's presenting antigens to lymphocytes).

Accessory cells (such as APC's) ingest antigens and partially digest them. Fragments of the antigens (containing the haptens) are combined with MHC-II proteins and then together they are inserted into the plasma membrane. Helper T lymphocytes can recognise the MHC-I protein-hapten complexes if they also have receptors that can bind to the hapten. Recognition (or when the receptor on the lymphocyte is a complimentary match to the presented antigen) triggers the release of several kinds of cytokine (interleukins). The interplay between cell-to-cell contact and chemical co-stimulation is what finally activates the B cell (or B lymphocyte).

The process of B cell stimulation results in mitosis of both the helper T cell and the B cell, with the formation of a clonal population of each. This results in a very large number of cells, all producing identical antibodies specifically against the presented antigen (Figure 9 – 1). Some of the B cells are further activated to become plasma cells, which provides a large enough group of cells to produce enough antibody to combat the antigen while some cells become the long-lived memory cells (see text below and captions in Figure 9 – 1).

Immunologic Memory

Primary and Secondary Responses

The first time the body is exposed to a particular antigen, the resulting immune response is mild and brief. The second time that same antigen is encountered,

however, the response is strong and long-lasting. This is an important aspect of the specific immune response. How strong and long-lasting the secondary response is can vary from antigen to antigen. Immunization against specific pathogens is aimed at establishing immunologic memory through repeated, controlled exposures to their antigens (with little or no chance of being infected).

Memory B and T Lymphocytes

Some of the activated B cells that are formed as part of the primary immune response become short-lived plasma cells that produce antibodies (IgM and IgG). Others persist as memory B lymphocytes. These cells are able to multiply and transform rapidly into plasma cells during a secondary response, when they produce some IgM and lots of IgG. In the activation of T lymphocytes (both helper and cytotoxic T cells), memory T cells are also formed.

Cellular Immunity

Cellular immunity and humoral immunity have several similar features . T cells have membrane proteins that bind only to a specific antigen, just as B cells do. T cells rely on displaying fragments of antigens combined with their MHC proteins. T cells must be co-stimulated by interacting with other cells that present the same antigen-MHC combination. An antigenic challenge causes the co-stimulated cells to release cytokines that stimulate single T cells to divide to form clones of helper T cells and cytotoxic T cells.

Cytotoxic T cells are the effector cells of cellular immunity. They can cause the death of antigen-bearing cells in several ways, but all require that they have close contact. This explains why T lymphocytes circulate through the blood, connective tissues and lymph - they are searching out foreign or abnormal cells. This immune surveillance is aimed at cells infected by viruses, bacteria or parasites and at tumor cells. Cytotoxic T cells also attack foreign cells that are present in blood transfusions and tissue transplants. People with organ transplants (such as heart transplants) must take immunosuppressive drugs to suppress the cytotoxic T cells and allow the transplanted organs to survive.

THE CRUSTACEAN IMMUNE SYSTEM

The crustacean immune system lacks the immune memory characteristic of vertebrates and have to rely on the innate or non-specific response. However, crustaceans are no different from the vertebrates in that their immune defense is largely brought about by the activities of specialized blood cells or hemocytes. These crustacean hemocytes carry out phagocytosis, encapsulation, produce antimicrobial substances to remove or neutralize foreign particles and infectious agents.

Hemocyte types

Based on their morphological appearance and their staining properties, different hemocyte types can be distinguished in crustacean blood. However, these hemocyte types do not fall into the same groups as those found in fish and other vertebrates. The three types of hemocytes that can be isolated from crustacean hemolymph are the *hyaline*, the *semigranular*, and the *granular* (see Table 9 –2 for summary of their functions).

Table 9 – 2 . Crustacean hemocyte types and their known biological functions

Hemocyte type	Functions			
	Phagocytosis	Encapsulation	Cytotoxicity	ProPO Activating System
Hyaline	Yes	No	No data	No
Semigranular	Limited	Yes	Yes	Yes
Granular	No	Very limited	Yes	Yes

The hyaline hemocytes have phagocytic ability. This cell type, which lacks granules, is found in decapod crustaceans, but apparently the relative number of this cell type vary considerably among species.

The semigranular hemocytes are characterized by the presence of a number of small granules in their cytoplasm making them resemble the granulocytes of vertebrates. These cells respond to microbial cell wall polysaccharides such as bacterial lipopolysaccharides (LPS) and the β-1,3 glucans of fungi. They also have the ability to encapsulate foreign particles.

The granular hemocytes are characterized by the presence of large vesicles or granules in their cytoplasm. This suggests a role in the production, storage and secretion of antimicrobial compounds. They do not have phagocytic activity and their ability to encapsulate foreign particles is limited. The primary role of granular hemocytes is to store prophenol oxidase, which is key compound in crustacean defense reactions. These cells can be induced to unload and activate the prophenol oxidase by the presence of B-1,3 glucans, peptidoglycans and LPS from microorganisms. Once activated, the phenol oxidase catalyzes the oxidation of phenols to semiquinones and quinones that are highly toxic to microbes due to their high reactivity.

Defense Mechanisms in Crustaceans

Phagocytosis

Phagocytic cells are found throughout the animal kingdom. In lower invertebrates they also serve a nutritive function and in higher phyla they become more specialized by assuming a defensive role against microbial infections. A

microorganism that has penetrated the exoskeleton and enters the tissues or the blood is immediately attacked by phagocytic cells that are specialized to engulf and digest particulate matter. In crustaceans, these are the hyaline hemocytes. Their primary function is to clear the body of foreign particles including virus, bacteria and fungal cells. Objects too large to be phagocytosed by one cell are trapped inside aggregates of hemocytes.

Nodule Formation and Encapsulation

When crustaceans are invaded by a large number of microorganisms that exceed the capacity of phagocytic cells, nodule formation or cell clumping occurs. The microorganisms become entrapped in several layers of hemocytes, and generally the nodule become heavily melanized because of the host's phenoloxidase activity.

When a parasite is too large to become engulfed by phagocytosis several hemocytes will then collaborate by sealing off the foreign particle from circulation. This process is known as encapsulation. The semigranular hemocytes are the first cell to react to foreign particles and to encapsulate any invading intruder. However, little is known about the mechanisms by which foreign particles or microbes are dealt with after being engulfed or encapsulated by hemocytes.

Cytotoxicity

Warm-blooded animals (higher vertebrates) produce a specialized white blood cell capable of killing tumor cells and cells infected by virus. Such cells are called natural killer cells. Among the white blood cells in the hemolymph of crustaceans there are populations of specialized hemocytes that, like the natural killer cells in mammals, have the ability to kill foreign cells, tumor cells and non-tumor target cells.

Lectins

Agglutinating substances or lectins are present in the blood of a number of different crustaceans. Lectins are proteins or glycoproteins that have the ability to recognize and bind to the carbohydrates on the bacterial or fungal surfaces. Lectins do not have catalytic or enzymatic activity, their action is simply to immobilize or agglutinate microorganisms and then mediate the binding between hemocyte surfaces and the microorganisms (or other foreign bodies) and thus function as an opsonin.

Antimicrobial Proteins or Peptides

The immune systems of arthropods also rely on the production of proteins and peptides that possess antimicrobial activity against a wide range of microorganisms. However, in crustaceans the presence and characterization of these antimicrobial peptides has been poorly studied until now. Recently, hemocytic proteins have been isolated in the crab, *Carcinus maenas* and a 6.5kDa antimicrobial peptide has been characterized. In the penaeids, three antimicrobial peptides have been isolated from the hemocytes and plasma of *Penaeus vanamei*. They have been fully characterized and their cDNA cloned. Based on

their biochemical and structural features, the three peptides do not belong to any group of peptides that have been hitherto described. The peptides were named penaeidins after the genus *Penaeus*. Studies on the role of the penaeidins in the immune system are still continuing, as are studies on the search and characterization of other antimicrobial peptides in shrimp.

The Clotting Reaction

Since crustaceans have an open circulatory system, wounds must be sealed immediately to stop blood loss and prevent the entry and distribution of microbes within the body. In the shrimp the clotting process requires the presence of plasma proteins and cellular components. The key plasma protein that constitutes the clot has been named clotting protein or CP. It appears to be present in relatively high concentration in the hemolymph. The clotting reaction in crustaceans differs from that of vertebrates because aside from tissue damage as a trigger for the clotting cascade, the presence of microbial LPS is also a stimulus for the release of transglutaminase from hyaline cells that triggers the clotting process.

The Phenol Oxidase System

Parasites and microbes can gain entry into a crustacean body through wounds or as contaminants in the food. Some pathogens like the fungi penetrate the exoskeleton by secreting proteases and exerting mechanical forces. The response to this invasion can often be seen as dark spots in the cuticle and the intruders will become brown-black. The cause of this is melanin which is one of the end products of the phenoloxidase system. The enzyme responsible for the formation of melanin is phenoloxidase (PO). This enzyme (PO) catalyzes the oxidation of phenols to quinines that subsequently polymerize into melanin. During the formation of melanin, transient oxidation products are also formed which are highly reactive and toxic to microorganisms. The phenol oxidase system is thus an important component of the crustacean immune system.

A crucially significant feature of the phenol oxidase system is that it is able to identify a real infection and it can only be switched on by signals that are uniquely associated with the physical presence of pathogens. Crustaceans use the lipopolysaccharides LPS and the β-1,3 glucan molecules, which are components of the cell walls of microbes, as the specific signals to activate the phenol oxidase system. Crustacean hemolymph contains specialized binding proteins that seeks out and binds to the LPS and glucans of microbes. Once these binding proteins have reacted with their target LPS and glucans, they then bind to a specific receptor on the hemocytes (both semigranular and granular) and induce degranulation and the release of the prophenol oxidase, which can be converted from its proform into the active enzyme (phenol oxidase) again upon contact with microbial LPS and glucans. These specific binding proteins, whose structures have already been elucidated, can also act as opsonins that stimulate phagocytosis. Thus the crustacean phenol oxidase system is exquisitely designed to be specific against microbes and to avoid metabolically costly and harmful false alarms.

IMMUNOSUPPRESSION

A distinct subset of T lymphocytes, called suppressor cells, exists in the immune system to turn down antigen-driven responses, and as a mechanism to maintain tolerance to self-antigens in the periphery. Thus the immune system has a self-regulating mechanism to modulate its reactions especially to cells and tissues of its own. However, there are many situations where the suppression of the immune system is unwanted and may eventually lead to disease.

A number of internal and external factors exist that can cause this suppression:

Stress Stress can have marked effects on the health of fishes. Stress can come in many forms such nutritional stress due to improper diet and feeding schemes; environmental stress brought about by poor water quality and physical stress attendant to handling, crowding or confinement. These types of stress can sometimes be unavoidable in intensive fish farming. Prolonged exposure to stress or even very brief stressful experiences can depress certain aspects of the cellular and humoral immune systems consequently lowering resistance to pathogens. Once stress is experienced, a cascade of neuroendocrine events follows that generally leads to elevation of the steroid hormone, cortisol, in circulation. This hormone and other stress hormones (e.g. catecholamines) can reduce the number of circulating leukocytes and antibody-producing cells, and depress macrophage activity and distribution of leukocytes in to various body compartments.

Metals Aluminum, arsenic, cadmium, chromium, copper, lead, mercury, nickel and zinc are heavy metals that have been shown to lower the non-specific and specific immune response in fish making them more susceptible to various viral and bacterial diseases.

Aromatic Hydrocarbons Polychlorinated biphenyls (PCBs) have been reported to have modulating effects on the immune response in fish ranging from no effect reduction of antibody producing cells and increased susceptibility to disease. Chlorinated dioxin (TCDD) partially suppressed the mitogenic response in trout. It appears that fishes may not be as sensitive to halogenated aromatic hydrocarbons as do the higher vertebrates, like mice, with regard to their humoral immune system.

Pesticides Endrin, malathion, methyl bromide, triclorphon, DDT, Bayluscide and tributylin have immunosuppressive properties ranging from reduced lymphocyte number and phagocyte activity to necrosis of the thymus.

Drugs Oxytetraxycline, the antibiotic most used by fish culturist in treatment of bacterial disease, has been long known to be immunosuppressive in fish, reducing

the numbers of antibody-producing cells. Regardless of the mode of administration (injection, feeding or bath) an immunosuppressive effect is evident. However, oxolinic acid, a more recent addition to the list of drugs for treating fish bacterial disease, was found to not have immunosuppressive properties when used at the therapeutic levels recommended.

BIOLOGICAL CONTROL

At the turn of this century, the concept and practice of biological control was developed by agriculturists to control insect pests. In agriculture, biological control is generally defined as the use of a specially chosen living organism to control a particular insect pest. It is basically the use of one chosen organism to control another organism. This chosen organism might be a predator, parasite or disease, which will attack the harmful insect. It is a form of manipulating nature to increase a desired effect. In agriculture, a complete biological control program may range from choosing a pesticide, which will be least harmful to beneficial insects, to raising and releasing one insect to have it attack another, almost like a "living insecticide".

The scope of the concept of biological control was later expanded to include all kinds of pests. Such as the use of different species of carps to control the proliferation of water lilies and other plant pests that choke ponds and rivers. More recently, the concept of biological control was further expanded to include all forms of biological manipulation of the host, the environment and the pest (or pathogen) to minimize or control infestation or infection. It is this expanded scope of biological control that we will discuss here and we will focus on the use of biological control in preventing, minimizing or eradicating disease in aquaculture stocks.

Avoidance of Pathogens

Aquaculture facilities that use intensive techniques for production often provide an environment for the cultured organism that is unnatural and stressful. A primary factor is crowding or over-crowding. Under this condition the fish or the cultured organism has to compete with each other for space and dissolved oxygen and are confronted with the stress of exposure to increased metabolites such as ammonia, carbon dioxide and dissolve or suspended organic matter. It is not surprising that disease outbreaks often occur in these facilities. An aquaculturist's main concern then is to eliminate all possible sources of these disease agents.

Pathogen-free water supply

A pathogen-free water supply is obtained through the sterilization of fresh water or seawater. Sterilization of in-coming seawater may control the number of potentially harmful microorganisms entering the aquaculture systems but not necessarily totally eradicate these microorganisms in the water. The most commonly employed methods for controlling the presence of these microorganisms

are by filtration, ultraviolet light treatment, ozonation and chlorination.

1. Filtration

   Ultrafiltration of water supplies through sterile 0.2µm membrane filters has the advantage of having none of the detrimental effects associated with the other methods of water sterilization. However, it could be a slightly more expensive method than the other three and that absolute retention of bacteria (e.g. 100%) will not necessarily be achieved.

2. Ultraviolet light

   Ultraviolet light at a wavelength of 254nm or within the range 240 to 280nm disinfects seawater but not sterilizes it. It reduces the reproductive capacity of bacteria and fungi and is therefore bacteriostatic (or fungistatic) rather than bactericidal. Organic matter in seawater may also be oxidized depending on the ultraviolet energy emitted. The efficiency of ultraviolet light treatment is dependent upon the amount of particulate material suspended in the water and of the presence of natural pigments seawater. To improve efficiency, it is advisable to reduce particulate matter content by mechanical filtration prior to UV light exposure.

3. Ozone treatment

   Ozone is a highly oxidizing form of oxygen, if used with caution; it can be a powerful means of improving seawater quality. Ozone dissociates rapidly in seawater to provide a highly active oxygen atom. Ozone will disinfect and sterilize seawater, oxidize organic material and oxidize toxic nitrite to less toxic nitrate. However, ozone is highly toxic to both man and the organism being cultured. It is also highly corrosive to aquaculture equipment, be it metal or plastic. The effective dosage of ozone can be affected by factors that also consume ozone such as: chemical oxygen demand, salinity, dissolved substances, and microbial and plankton densities.

   The equipment manufacturers provide guidance on the use of ozonators and UV light cartridges.

4. Chlorination

   Chlorination, although less effective than ozonation, is the more popular form of seawater sterilization in aquaculture facilities in Southeast Asia. This could be due to the fact that chlorination does not involve special equipment. However, it shares many of the disadvantages of ozone in that it is toxic to both man and the cultured organisms, corrosive, and may form toxic stable complexes with organic compounds (e.g. chloramines). Chlorine as calcium or sodium hypochlorite is commonly used in hatcheries in south Asia.

Pathogen-free diets

Most manufacturers of artificial diets claim that their products are pathogen-free. In most cases the preparation of commercial feeds involves high temperatures of about 120°C for short periods that should be enough to pasteurize if not sterilize the feed.

On the other hand, the feeding of live food may present a different set of problems in that some germicides that are used to disinfect live feed could also be toxic to the live food organism. In Japan, live foods are managed under sanitary conditions to prevent intestinal infections of larval fish and shellfish. Bath treatments with a nitrofuran derivative, sodium nifurstyrenate, in live diets, such as rotifers and brine shrimp, are effective approaches to decreasing the number of bacteria in these diets.

Specific-pathogen-free (SPF) stocks

SPF animals are defined as "animals that are free of specified microorganisms and parasites but not necessarily free of others." Although specific pathogen free technology has been practiced in agriculture for many decades it has only been recently applied in aquaculture.

In prawn farming, some pathogens causing epizootics during the grow-out phase have been traced to be vertically transmitted from wild broodstock that are carriers of diseases. The vertical transmission of pathogens from mother to larvae is a continuous threat to production. In Taiwan, the screening of wild broodstock and the production of captive reared broodstock, which are certified SPF with regards to white spot syndrome virus (WSSV), have resulted in grow-out cycles free of WSSV epizootics. Although the use of SPF broodstock does not result in disease resistant or even disease tolerant stock, it is proving to be one effective managerial control measure, which minimizes the likelihood of epizootics due to an identifiable pathogen.

Disease inspection, quarantine, and international regulations

A fish health inspection is a procedure by which a sample of fish collected from a defined fish population and examined for the presence of certain specific pathogens. Knowledge of the presence of pathogens can be used to prevent the introduction of serious fish diseases into areas where they do not presently occur and to better manage those diseases.

A number of serious diseases are caused by organisms that can survive only for very short periods of time outside of the fish they infect. These organisms are called *obligate pathogens*. Fish health inspections are conducted to detect this obligate pathogen group because one of the most likely methods by which these are spread to new areas is through infected fish. Another broad group of pathogens are called *facultative pathogens*. They are commonly found in all aquatic environments and may cause disease only when the host fish is stressed. Fish health inspections are not typically conducted to detect organisms in the facultative group.

Quarantine is defined as the holding or rearing of animals under conditions that prevent their escape or the escape of a disease agent. Quarantine can provide a useful environment for "filtering out" disease in new stocks, especially if cures are available. Unfortunately post-treatment checks of individuals in large aquatic animal populations are generally not practical, and few cures are known for important fish and shrimp diseases. Quarantines are sometimes used as a means to avoid a particular disease agent. Such a concept often emerges as a regulatory effort to establish "disease free" or "pathogen free"

status of imports. While it is true that the quarantine technique is a means for disease avoidance, there is a great deal of difference between the presence of disease and the presence of pathogens.

A number of international organizations have been developing guidelines or codes of practice for the introduction and transfer of species. These guidelines establish specific diagnostic techniques for pathogens, define sanitary regulations of the individual countries, and develop health certificates for facilities to accompany shipments and prohibit the international transfer of aquatic animals that are not accompanied by these certificates. The organizations that have developed protocols include the Office International des Epizooties (OIE), the European Inland Fisheries Advisory Commission (EIFAC), and the International Council for the Exploration of the Sea (ICES). The ICES code of practice details how the species approved for importation are to be handled, the following are the steps of the protocol: 1) imported stocks are examined for potential pathogen or parasites, 2) stocks that are pathogen-free are grown into broodstock in an approved quarantine facility where they are regularly examined for pathogens, 3) if no pathogens are detected, the first generation offspring are released to the farmer, the original imported stock never leaves the quarantine site and disease studies are continued on the transplanted individuals, and 4) to eliminate the need for further importations of this species, it is recommended that F_1 individuals be used to establish a local broodstock.

Biology of the pathogen

The control of disease in an aquatic environment is particularly unique because water, as a universal solvent, makes prevention and control of physical, chemical and biological contamination of water and water sources much more difficult compared to land-based agriculture. Aside from the water itself, aquatic animals in all stages of their life cycles are carriers and reservoirs of disease.

Knowledge on the biology of the pathogen is one tool in breaking the cycle of infection and transmission. In shrimp hatcheries, for example, there is evidence that the virulence of facultative pathogens (e.g. *Vibrio harveyi*) increases from cycle to cycle. Successive passages from host to host provide a mechanism where the characteristic of virulence is selected. In order to limit the spread, adaptation and selection of these virulent strains, it is advisable to separate each group of larvae in time, (such as by batch culture, or by giving rest periods to the whole facility) and to isolate each group in space (by giving extra distance between tanks or using enclosures in some tanks). Any vector of contamination (e.g. water, workers, equipment and the fish themselves) must be controlled continuously to prevent vertical and horizontal transmission of pathogens. Another example is in the use of antibiotics in hatcheries. The use or overuse of antibiotics in hatcheries and farms may encourage outbreaks of oomycetes (*Lagenidium*, *Sirolpidium* and *Haliphthoros*) by removing the competitive bacterial microflora allowing these organisms to proliferate. An additional example where knowledge of the physiology of the pathogen can be put to good use is in cases of vibriosis. The *Vibrio* species that infect marine fish and shellfish are not tolerant to freshwater or very low salinities. If the infected cultured stocks happens to be euryhaline like the sea bass then the salinity of

the rearing water can be lowered gradually even down to zero parts per thousand salinity to eliminate the pathogen.

The Role of Stress in Disease

Because of the high stocking densities used in most aquaculture operations and the scarcity of water supplies free of aquatic microorganisms, a normally functioning immune system is absolutely essential to the health and physiological balance of the fish being cultured. The crowded conditions may increase initial susceptibility to infections and facilitate the horizontal or fish to fish transmission of pathogens when infectious disease outbreaks do occur.

Stress is defined as physical, chemical or biological factors that cause bodily reactions that may contribute to disease and death. Many potential fish disease pathogens are continually present in the water, soil, and air or in the fish themselves and outbreaks of clinical disease usually occur only when the fish is under some form of stress.

Aquaculture practices that increase stress are:

1. High stocking densities and poor water quality
2. Injury during handling (e.g. chasing, netting, sorting and shipping)
3. Improper nutrition
4. Poor sanitation

Some managerial practices that may help prevent stress:

1. Water quality
    - Do not exceed the carrying capacity of ponds and tanks.
    - Regularly monitor water quality parameters.
    - Prevent the accumulation of organic debris, nitrogenous wastes, carbon dioxide and hydrogen sulfide.
    - Maintain the appropriate or optimal pH, alkalinity, temperature and salinity for the species.
2. Handling and transporting
    - Exercise speed and gentleness when handling fish.
    - Use knitted mesh nets rather than knotted nets or better still, use smaller mesh size nets rather than larger mesh to reduce scale loss or entanglement of fins and finrays during capture.
    - Minimize the number of times that fish are handled or lifted from water and if possible use anesthetics to slightly sedate the fish.
    - Harvest, handle and transport fish at the point of their life cycle when they are least susceptible to stress, and in warm areas handle fish only during cooler periods of the day and add ice to the transport water to decrease fish metabolism and increase oxygen solubility.
    - Maintain high levels of oxygen as this is critical for the rapid recovery of fish from the struggle of capture and handling; for freshwater fish add salt at 0.3 to 1.0 percent in the transport water to minimize osmotic stress and bacterial infection.

3. Nutrition

   - Feed high quality diets that will meet the specific nutritional requirements of the species as different species have different levels of requirements for fatty acids, amino acids etc. and feeds that do not meet these needs are simply metabolized leading to increase excretion of wastes instead of being used for growth.
   - Use proper feeding rates and feeding schedules.
   - Store feeds in a cool dry place to preserve nutrients and prevent the growth of toxin producing fungi.

4. Sanitation

   - Quarantine all new fish.
   - Make sure that water supplies are not contaminated from the source.
   - Immediately remove all dead fish and dispose them properly to prevent the spread of diseases.
   - Observe good sanitation processes by disinfecting containers, nets and equipment to minimize transmission of parasites and disease from one population to another.

Improvements in Host Resistance

Dietary Enhancement

The resistance of fish to diseases involves a complex array of mechanisms that include maintenance of epithelial integrity and mucus coat, non-specific cellular factors such as phagocytosis by leukocytes, non-specific humoral factors such as lysozyme, complement, and transferrin, and specific humoral and cellular immunity. A variety of nutritional components can influence the incidence and severity of a number of infectious diseases. Some micronutrients that are known to enhance disease resistance include vitamins C, B_6, E, and A, and the minerals iron and fluoride. The role of the macronutrients (protein, lipid, and carbohydrate) in disease resistance has not yet been clearly defined. There are evidences that certain fatty acids may have essential roles in disease resistance.

Artificial diets that produce the best growth performance may not necessarily produce the optimal immune status. In marginal deficiencies, the fish continue to grow, appear healthy, and show no gross or histopathological signs of disease, yet significant depression of disease resistance is present and disease outbreaks become evident only when the fish are subjected to the slightest stress.

Increased levels of certain macro and micronutrients may be beneficial before and during the exposure to certain disease agents, but may be detrimental in other instances. Nutritional enhancement of disease resistance is a new field of research yet despite the need for more studies; the potential for dietary enhancement of disease resistance certainly exists.

Improving Genetic Resistance to Disease

Disease resistance can also be achieved by genetic improvement of cultured stocks. Research to improve genetic resistance to disease in farm animals has been in progress for some years now. Loses due to diseases in farm animal production are estimated at 10 to 20% of total production values. In aquaculture this figure could reach 100% during disease outbreaks. Thus breeding programs that improve disease resistance may significantly increase production values.

Selective breeding for disease resistance in fish and shellfish was given emphasis only recently. Most of the work done in fish were at first focused on improved growth rates, control of maturation, maintenance of genetic vigor and other characteristics like color (e.g. Red Tilapia), later studies then included selection for disease resistance. There are three main strategies that can be used for the improvement of disease resistance in farm and aquatic animals, namely: conventional selective breeding programs based on morphological traits; marker-assisted selection utilizing associated DNA polymorphism; and transgenic approaches.

Genetic selection for pathogen resistance in cultured shrimp species is given more attention by shrimp farmers because shrimp, unlike fish, cannot be efficiently vaccinated due to their lack of a lymphoid system that produces antibodies to the antigens of disease agents. The international shrimp breeding program has come up with a list of quantifiable immune traits for individual selection, namely: hemograms (or blood profile), hemocyte respiratory burst (related to killing ability of hemocytes), plasma antibacterial activity, levels of plasma coagulogen, and immune index (total of these quantifiable immune traits). These indexes can easily be determined by getting a sample of blood from the shrimps. Individuals scoring high in many or all these quantifiable traits can then be selected as the breeders in a breeding program.

Vaccination

Cells with the same morphology as the lymphocytes in warm-blooded animals are also present in the spleen, thymus, kidney, and blood of fishes. It is also possible to distinguish between B and T cells in fish as they are identified in higher vertebrates. Vaccination in fish is therefore possible and specific vaccines can be developed along the same principles as for warm-blooded animals. It appears that the thymus organ in young fish and the head kidney are the primary organs of lymphocyte differentiation in fish, whereas the spleen is a secondary lymphoid organ that harbors both B and T cells. The hemocytes of crustaceans do not include any cell types with properties comparable to B and T cells. Although “vaccines” for shrimps in aquaculture are being developed and marketed, such products do not fall within the current definition of a vaccine. If they reduce disease, it may be the result of a non-specific stimulation of the hemocytes by the cell wall fragments from the bacteria used in the “vaccine,” or by the adjuvants it contains and not due to a specific antibody development against the disease,

Reports on the capability of fish to produce antibodies against bacterial pathogens first came out in 1935. Then in 1942, it was demonstrated that this anti-

body response in fish translated into a protective immune response. What followed, however, was a long period of disinterest in vaccines due to the fascination of the new antimicrobial compounds (antibiotics) that came on the market immediately after the Second World War. It was only in the mid to late 1970s that attention was again given to vaccination as a means of preventing and controlling fish disease and the development of commercially available vaccines. The reasons for these turn of events were varied: the high cost of using chemotherapy, the short-term nature of the protection obtained with antibiotics, the increasing incidence of antibiotic resistant fish pathogens, and environmental concerns on the use of antibiotics.

1. Vaccine Development

Bacterial Vaccines. A number of vaccines are currently being developed against bacterial fish pathogens. Most of these works are focused on salmonid pathogens and a few are being directed against bacterial pathogens in carp and catfish. Japanese researchers are focusing their work on their cultured fish especially the yellowtail for the development of vaccines against streptococcal and *Pasteurella* infections. To date, there are still no commercially available vaccines for bacterial diseases in warm water fish.

Viral Vaccines. For some years, the only commercially available vaccine against a fish virus was the spring viraemia of carp (SVC), caused by *Rhabdovirus carpio*. It was administered by injection since the disease affects carp at a size and age (9-12 months) when they are easy to handle. However, the same is not applicable for the other important fish viruses such as infectious pancreatic necrosis virus (IPN), viral hemorrhagic septicemia (VHS) virus, infectious hematopoetic necrosis (IHN) virus, and channel catfish (CC) virus. These cause severe mortalities in fish during the fry stage in which injection is not practical. Immersion in a suspension of inactivated virus has given unsatisfactory results. Another approach had been tried using live attenuated virus or avirulent forms of the virus. Although reasonable protection has been achieved using this approach, it has been abandoned due to concerns on residual virulence in target species, virulence in non-target species, and persistence in the treated fish leading to the fear that the virus might back-mutate to virulence.

Parasite Vaccines. Parasitologists have only recently exploited the immune system to protect fish against parasitic disease. Vaccination techniques are being developed against parasitic protozoans by intraperitoneal injection of live attenuated parasites. Some evidence shows the passive transfer of protective immunity against these parasites from immune to naïve fish, and to egg. Studies are also in progress in the development of vaccines against helminthic and copepod parasites.

2. Types of Vaccines

Inactivated. The pathogenic organism is cultured *in vitro* usually in broth then killed using heat or formalin.

Live Attenuated. When the approach of using inactivated pathogens fails to elicit an immune reaction then live attenuated pathogens are used. Removing some genes from the pathogen, which is usually, a virus, making it

non-virulent develops attenuated strains. There is always the danger of back-mutation to the virulent wild type.

Recombinant. The antigens in pathogenic organisms are just minor portions of their structures, such as cell wall proteins or part of the protein coat of viruses. The genes that code for these antigenic structures can be isolated and inserted into yeast or bacterial DNA (e.g. *E. coli*) where they become incorporated and expressed in large amount. The products, usually proteins, are then harvested from the broth of cultured recombinant yeast or bacteria and used as vaccines.

DNA. The most recent approach to vaccine design is by genetic immunization or the injection of naked DNA of a pathogen into the muscle of the host. The DNA, which usually encodes a single gene of the pathogen, is expressed extrachromosomaly in the muscle cells. The newly synthesized antigen can then stimulate the immune defense of the host conferring the host with life long immunity.

3. Modes of Vaccination Delivery

The route vaccine administration depend largely on the species of fish, the size, the husbandry, the disease, the stage of the life cycle of the fish.

Direct Immersion. Vaccination by immersion is commonly practiced with very small fish where it is a convenient and highly cost-effective method of vaccine administration. Antigen uptake takes place mostly through the gills, although some may be taken up through the skin, and the lateral line and some are swallowed.

Spray Administration. Spraying a solution of a vaccine is a variation of the immersion method suitable for larger fish than those given immersion treatment. Fish are run through a conveyer belt under two or more jets containing the vaccine at 1:10 dilution for not less than 10 seconds. Antigen, like the immersion technique, is taken up through the gills.

Peroral Administration. The oral route of administration of vaccine as feed additive should have many clear advantages like the absence of handling stress, no scars on the fish to lower its value, the freedom to choose vaccination dates, no safety risk to the operator, and no risk of spreading infection through needles. However, to date, there are no cost-effective vaccines that can be administered orally.

Injection. This is the route of administration commonly used throughout the salmon industry. A single injection can provide a high degree of protection through the whole length of the culture period or production cycle. The added advantage of this method is that the process allows fish to be graded, counted, and monitored for abnormalities and signs of disease.

Immunostimulation

Immunostimulants are chemical compounds that activate the immune system of animals and increase their resistance to infectious diseases. It has been known for many years that cell wall fragments when introduced into animals will render them more resistant to pathogenic diseases. The ability of the im-

mune system to respond to microbial surface components is the result of an evolutionary process whereby animals have developed mechanisms to detect common and highly conserved chemical components of pathogenic organisms and to use these chemical components as "alarm signals" to switch on the defense mechanisms against infection. The immune system will therefore respond to an immunostimulant as if challenged by a pathogenic organism.

Immunostimulants offer many advantages when used in fish farming: 1) they may be used alone, inducing elevated activities of the non-specific defense mechanisms; 2) they promote a more effective immune response to pathogens; 3) they enhance the level and duration of the specific immune response, both cell-mediated and humoral, following vaccination; 4) they overcome the immunosuppressive effects of stress and of those pathogens that damage or interfere with the cells of the immune system.

Immunostimulants may be used prior to situations known to result in stress (handling and transfer, crowding, poor water quality, etc.) or during development stages when the animals are more susceptible to infectious diseases (larval phases, maturation and spawning, etc.). Larval or very young fish and crustaceans at all stages of their life cycle do not posses the specific (or adaptive) immune system and largely rely on nonspecific cellular defense functions to resist infections. The use of immunostimulants could improve growth and survival of juvenile fish and crustaceans. Another field where immunostimulants might be of use is in the application of antibiotics to combat infectious diseases. Most antibiotics have been proven to be immunosuppressants. A combined administration of immunostimulants and antibiotics may counteract this suppressive effect.

Substances with Immunostimulatory Effect

1. Bacterial products - these are usually cell wall components of bacteria (e.g. glycoproteins and lipopolysaccharides)
2. Products from mycelial fungi - the immunostimulants derived from mycelial fungi are all glucose polymers (e.g. lentinan, schizophyllan and scleroglucan)
3. Yeast cell wall products - these are structural components of yeast cell wall (e.g. zymosan, ß-1, 3-glucans)
4. Soluble and particle bound ß-glucans - these are animated ß-1, 3-glucans or soluble ß-1, 3-glucans bound to microbeads
5. Glycans - polysaccharides also containing sugars other than glucose
6. Chitosan – extracted from the exoskeleton of shrimps and other crustaceans
7. Peptides from animal extracts - examples of these are peptones and protein concentrates from fish, peptides extracted from the thymus of animals, the compound EF-203 from chicken eggs
8. Unspecified extracts - examples under this category are extracts from a tunicate, a peptidoglucan extract from *Bifidobacterium thermophilum*, extracts from the seeds of "malunggay" (*Moringa oleifera)*

9. Synthetic compounds - these are usually dipeptides or lipopeptides extracted from microorganisms as well as other chemical compounds that are incidentally found to have immunostimulating properties (e.g. the antihelminthic drug levamisole)

10. Cytokines - these are the molecules involved in the transmission of signals between leukocytes, such as interleukins, interferons, tumor necrosis factors, colony-stimulating factor and monocyte chemotactic factor and have dominated research in immunotherapy in humans

Biological Modification of the Culture System

Biological Filtration

Biological filters facilitate the purification of the water in high density, semi-closed or closed aquaculture facilities by the oxidation of ammonia to nitrite and nitrite to nitrate. Ammonia oxidation is accomplished by *Nitrosomonas* while nitrite oxidation to nitrate is completed by *Nitrobacter.* Both nitrite and ammonia are highly toxic compounds. The well being of the animals in the culture system depends on the ability of the biofilter to rapidly convert ammonia to nitrate which is relatively less toxic.

Ammonia is a ubiquitous by-product in an aquatic environment. It is the main excretory product of water-breathing animals. It is also the end product of the decay of organic matter. In newly established closed system the accumulation of dangerous levels of ammonia and nitrite might occur since it takes time for the biofilter to become completely colonized by *Nitrosomonas* and *Nitrobacter*. Convenient sources of nitrifying bacteria are rich garden soil (for freshwater facilities) and gravel from existing well-established biofilters. Tap water or unfiltered seawater contains these bacteria but only in small numbers. Commercial bacterial inoculates for the biofilter are also available.

Probiotics

Historically, probiotics are a group of food and feed products for both human and animal consumption and are also known as direct fed microbials. A Russian scientist who attributed the longevity of a group of Bulgarians to their consumption of fermented milk products (yogurt) first wrote the concept of probiotics about in 1908. In 1960, an Oregon microbiology professor first used the term “probiotic,” meaning for life as opposed to “antibiotic,” or against life.

In aquaculture, commercially available probiotic products contain bacterial inocula not for consumption of the fish but for the environment. Species of *Bacillus* are most commonly used, but species of *Nitrobacter, Pseudomonas, Enterobacter, Cellulomonas, Rhodopseudomonas*, and photosynthetic sulfur bacteria or their combination has been used as inocula. Some probiotic products contain enzymes or plant extracts without live bacteria.

Manufacturers of probiotics for aquaculture claim that the mode of action of their products is to enhance natural processes such as organic matter degradation, nitrification, ammonia removal, denitrification, sulfide oxidation, and degradation of toxic pollutants. They further claim that increasing the abun-

dance of useful bacteria, competitive exclusion of undesirable species, including pathogenic ones, occurs.

Polyculture

The practice of keeping several of different species in the same pond is polyculture. One of the objectives of using polyculture is to better utilize available foods in the pond. Another objective would be to control unwanted competing offspring of a cultured fish by providing a predator species. Polyculturing fish and prawns with filtering organisms to reduce phytoplankton, bacteria, and organic particles had been done on the experimental scale. Oyster, clams, macroalgae, and bloodworms are often utilized to accomplish this clean up. It is also suggested that a pond or tank containing a more varied population of fish or shellfish will also harbor a more diverse bacterial flora. This diversity may prevent the dominance of any bacterial species specially the opportunistic facultative pathogens.

Phage Therapy

Many antibacterial agents have through time aided the human race in fighting bacterial diseases. In the last part of the 1800's and in the early 1900's scientists have discovered viral particles that had a killing effect on bacteria. These particles were named bacteriophage, or phage, for their ability to "eat" bacteria. Phage therapy is the use of bacteriophage to treat bacterial disease. The development of antibiotics in the 1940's directed attention away from bacteriophages as a mode of treatment. However, the recent concern regarding bacterial resistance to antibiotics has prompted a renewed interest in bacteriophage as biological control of bacterial pathogens.

Each kind of bacteria has its own phages, which can be isolated wherever that particular bacterium grows - from sewage, feces, soil, the sea, ocean depths and hot springs. Phages are specific to their bacterial host because of a particular protein antibody on the surface of the bacteria that a phage will specifically bind to. Since phages are specific they cause much less damage to the normal microbial balance of the host. Moreover, phages replicate within the body of the infected animal thus requiring only a single dose of phage that multiply only as long as the target bacteria are present.

Most of the work on phage therapy was conducted in Eastern Europe and all these concentrated on common human bacterial disease. Although phage therapy is still not practiced in agriculture and aquaculture, it shows great potential as a safe, specific and cheap alternative to chemotherapeutics.

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