Measurement of the Innate Cellular Immune Responses of Hybrid Striped Bass and Rainbow Trout

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Introduction

Healthy fish are required for optimal aquaculture production. In addition to needing good nutrition and adequate water quality, fish need to be protected against infectious disease. The health of fish is maintained by the immune system, which is susceptible to a variety of stressors in the aquaculture environment.

The immune system of fish and other vertebrates can be divided into non-specific and specific arms, and each of these has a separate role in the destruction and removal of invading pathogens. The non-specific, or innate, arm of the host immune system provides an immediate and relatively broad response to a variety of invading pathogens, even in the absence of prior exposure. The cells of the non-specific immune system are also instrumental in the activation of the specific immune response. As its name implies, the specific immune response narrowly targets its actions to a pathogen that has infected the fish or to vaccination with antigens from that pathogen. Because fish are poikilothermic (cold blooded), their specific immune system is slower to respond at cooler temperatures. Therefore, they tend to rely on their innate immune system to maintain health more than warm-blooded animals such as mammals or birds.

The first portion of this publication provides an overview of the immune system of boney fish. The second portion describes three cellular assays developed for the innate immune system of salmonids (Oncorhynchus sp.), which have also been adapted for use with hybrid striped bass (Morone chrysops × M. saxatilis). In addition to serving as research tools, these assays can provide a good indication of overall disease resistance, and this information can be used by fish culturists to optimize rearing conditions and increase the health of the stock.

Fish immune system

Access to the interior of fish by pathogens (bacteria, viruses, fungi, parasites, etc.) in the surrounding environment is blocked by a number of intrinsic factors. These include, but are not limited to, the mucus, scales, and skin on the surface of the fish; the acidic conditions in the stomach; and the normal bacterial flora in the intestine. If a pathogen is able to cross one or more of these barriers and penetrate the fish’s body it then faces the immune system of the fish.

The immune system has evolved to discriminate between self (e.g., host tissues) and non-self (e.g., pathogens, foreign bodies). Recognition of pathogens by the immune system is made possible by molecules on the pathogen that are dissimilar to any self-molecules. Some of these molecules are invariant or are present in most members of a class of pathogens and are referred to as pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include double-stranded RNA of viruses, the peptidoglycan of Gram + bacteria, the lipopolysaccharide of Gram – bacteria, and unmethylated DNA.

PAMPs are generally recognized by members of the non-specific arm of the immune system and can elicit an immune response. Each pathogen also has a variety of molecules that are unique to that organism. Some portions of these pathogen-specific molecules can be recognized by cells of the specific immune system. When these portions of the pathogen’s molecule, termed antigens, are recognized by the specific immune system, they can elicit a strong immune response against that particular pathogen.

Overview of non-specific immune system

The non-specific immune system provides an immediate response to an invading pathogen, and is composed of both a humoral arm and a cellular arm. The humoral arm is a diverse array of defensive molecules that are found in the serum, mucus, and eggs of fish. The complement system is composed of several proteins whose sequential activation leads to the formation of pores in the pathogen’s cell membrane. Complement is activated by the binding of antibodies to the pathogen surface (classical activation pathway) or by intrinsic factors of some pathogens (alternative pathway). Activation of either complement pathway also releases several proteins important in inflammation.

These proteins have a variety of functions, including
attraction of macrophage cells and providing a “handle” on the surface of the pathogen to enhance ingestion by phagocytic cells, a process called opsonization. Lysozyme is a low molecular weight enzyme that breaks down the peptidoglycan layer of bacteria. Interferons are proteins produced by virus-infected cells that inhibit the replication of viral pathogens in surrounding cells. C-reactive protein binds phosphorylcholine, a cell-wall component of many bacteria, fungi, and parasites, and has been shown to activate complement and enhance phagocytosis in some species of fish. Fish also produce a variety of lectin molecules, each of which binds a different combination of carbohydrates on the pathogen surface. Because most pathogens require iron to establish an infection, host transferrin proteins sequester the iron so that it is unavailable to the pathogen. Taken together, these non-specific humoral immune factors provide an unfavorable environment to any invading pathogen.

The cellular arm of the non-specific immune system is composed of several cell types, including macrophages, granulocytes, and non-specific cytotoxic cells. The first two cell types are particularly important in inflammation; they migrate to sites of pathogen infection and are then active in pathogen destruction. When they respond to an infection, macrophages perform a number of functions, including migration to sites of infection, phagocytosis of pathogens, destruction of pathogens, and presentation of pathogen molecules to cells of the specific immune system.

Granulocytes refer to several cell types that contain cytoplasmic granules. The group includes polymorphonuclear neutrophils (PMNs), eosinophils, and basophils in a few fish species. PMNs are also important in containing a pathogen during an inflammatory reaction, and share functions with macrophages. Non-specific cytotoxic cells recognize tumor cells and some parasites in vivo.

Overview of specific immune system

The specific immune system responds to an invading pathogen and then reacts in an appropriate manner to eliminate that specific invading organism. Upon repeated exposure to the pathogen, the specific immune system produces a faster and more robust response. The primary cell types involved in specific immune responses are lymphocytes. Although morphologically similar—small cells with little cytoplasm—the lymphocytes can be functionally divided into cytotoxic T cells, helper T cells, and B cells. On T cells, the antigen receptor, termed the T-cell receptor, only recognizes antigen when presented on the surface of another cell in association with a group of host proteins called the major histocompatibility complex (MHC). The antigen receptors of B cells are cell surface bound forms of the specific antibody that cells will produce if activated. When host cells are infected with a virus, they usually present portions of some of the viral proteins on their cell surface in context with the type 1 MHC for recognition by cytotoxic T cells. The cytotoxic T cells then engage and kill the infected host cell. The end result of an immune response involving helper T cells and B cells is the production of antibodies.

Antibodies are protein molecules produced by activated B cells that attach to antigens of the pathogen. Once antibodies attach to the pathogen, they can activate complement to lyse the pathogen or make it easier for macrophages to ingest the pathogen. Peak production of antibodies against an invading pathogen usually takes several weeks, although this is dependent on the fish species and the water temperature.

To produce an antibody response to an invading pathogen, both B cells and T helper cells must recognize molecules of that pathogen through their respective antigen receptors. When a pathogen infects a fish, some of the invaders are phagocytized by macrophages or other non-specific immune

Figure 2. Diagram of the immune system (http:/ /pathmicro.med.sc.edu/ghaffar/innate.htm)
system cells. After ingestion and killing, molecular portions of the pathogen are presented on the cell surface in association with the type 2 MHC, which is recognized by helper T cells. T cell receptor binding, along with cell signals (cytokines) from the antigen presenting cell, activate the helper T cell, which multiplies and begins producing its own set of cytokines.

Concomitantly, certain B cells will also bind to the pathogen by their surface-bound antibody. The combination of multiple surface antibodies binding antigen, interaction with a helper T cell via the presented antigen, and reception of the helper T cell cytokines activates the B cell. The activated B cell multiplies and the clones produce large amounts of soluble antibody.

Since both the helper T cell and the B cell are recognizing foreign molecules from the invading pathogen, the antibody response is specific to that pathogen. Furthermore, some of the expanded B and T cells enter a quiescent state and become memory cells. Memory cells can be readily activated upon re-infection of the fish with the same pathogen. The decreased amount of time needed to react to a second infection, and the increased secondary response is the reason for vaccination against typical diseases.

**Macrophages—**

**Key players in the immune response**

After their production in the hematopoietic anterior kidney, monocytes circulate briefly in the bloodstream before maturing into macrophages and migrating into the fish’s tissues. Neutrophils circulate with the blood but quickly emigrate into tissues in response to invading pathogens. Both of these phagocyte types have a variety of receptors on their surfaces, including receptors for PAMPs, activated complement components, and antigen-bound antibody.

When a phagocyte encounters a pathogen, three separate activities occur. The first involves the attachment, uptake, and destruction of the pathogen. Once the pathogen has adhered to the surface of the phagocyte, it is ingested by a process known as phagocytosis. The cell membrane of the phagocyte invaginates and surrounds the foreign invader so that the pathogen is contained within a cytoplasmic vacuole known as a phagosome. Other vacuoles within the phagocytic cell, called lysosomes, contain enzymes and reactive oxygen species (ROS; superoxide anion, H₂O₂, etc.). The lysosomes fuse with the phagosome to destroy the internalized pathogen.

The second activity is the release of a number of cytokines by the phagocytic cells. The release of these cytokines leads to the development of the inflammatory response. In an area of inflammation, the local blood vessels dilate so that blood plasma can flow into the area, bringing complement, antibodies, and other humoral defensive factors. There is also a large migration of phagocytic cells to the site—neutrophils arrive first, and later the monocytes/macrophages appear. Activated phagocytes release bactericidal enzymes and ROS to kill extracellular pathogens. In later stages of inflammation, the blood in the local small vessels coagulates to prevent the escape of the pathogen into the general circulation.

The third activity of the activated phagocytes is their functioning as antigen presenting cells to initiate the specific immune response. Pathogens processed through the phagosome are broken down and their antigens are presented on the phagocyte cell surface in association with the type 2 MHC. These can be recognized by T helper cells as described above.
Due to the central roles the phagocytic cells play in the specific and nonspecific arms of the immune system, the relative ease in collecting these cells, and the numerous cellular functions that can be measured, these cells are of high interest in monitoring the immune systems of fish.

A wide variety of leucocytes can be obtained from the hematopoietic anterior region of the kidney. After removal and disruption of the anterior kidney, the resulting single cell suspension can be centrifuged over a discontinuous density gradient to enrich for macrophages and granulocytes. The macrophages can be further purified by allowing the cells to adhere to a glass dish or tissue culture flask, then removing the nonadherent cells. The isolated cells can be assayed within a few hours of removal from the fish and are usually not in an activated state when taken from a healthy individual. A fairly pure sample of activated macrophages and neutrophils can also be obtained by taking advantage of the ability of these cells to migrate to the site of an infection. An immunogen is injected into the peritoneal cavity of the fish. Neutrophils are the first cells to migrate into the cavity, followed by large numbers of activated macrophages. These cells can be harvested by lavaging the peritoneal cavity a few days after injection of the immunogen.

Purified or enriched macrophage and granulocyte preparations have been extensively studied in fish, and a wide variety of assays have been developed to assess them. A brief list includes assays to evaluate macrophage migration (Afonso et al. 1998), response to cytokines (Neumann et al. 1995), adherence (Saggers and Gould 1989), phagocytosis (Alcorn et al. 2002, Li et al. 2004), killing of internalized bacteria (Skarmeta et al. 1995), and antigen presentation (Vallejo et al. 1992). Researchers working on the WRAC Immunology Project have been optimizing macrophage assays to study the effects of hatchery practices on the immune system of rainbow trout and hybrid striped bass. The development of these assays has been guided by the desire to test the cells as quickly as possible after removal from the fish so that in vitro culture conditions do not have time to affect cell health. The assays were also developed to automate the data collection process so that subjective human measurements are reduced and sample numbers and/or sample size can be increased for improved statistical power.

Three assays are described below, each of which investigates a different phase of the monocyte/macrophage’s ability to combat an invading pathogen: (1) migration of the cells to the site of infection; (2) phagocytosis of the invader; and (3) intracellular killing of the pathogen.

**Macrophage migration**

As described above, phagocytic cells migrate to the site of an infection. Once in the tissue, the phagocytes migrate up a gradient of cytokines and other factors diffusing out from the site of inflammation. In this assay, the phagocytes migrate through a porous membrane towards fish serum whose complement has been activated by a yeast cell wall product (zymosan).

The migration assay that was used for these studies was based on the semi-automated fluorometric method of Frevert et al. (1998). Each phagocyte preparation is incubated with a compound that becomes fluorescent once it diffuses into the cells. The stained cells are washed and resuspended in cell culture medium (L-15). The stained anterior kidney cells are then placed on a porous membrane immediately over the wells of a 96-well microplate. The wells of the microplate were previously filled with either L-15, or L-15 containing activated fish serum. Also, some wells were loaded with live stained cells to represent the fluorescence signal if all of the cells migrated through the membrane (total fluorescence). The plate is incubated for 2 hours, allowing the cells on the top of the membrane filter to migrate into the lower wells. A microplate fluorometer is used to measure the fluorescence of each well from below so that only the fluorescence of cells that had migrated through the membrane is quantitated. The fluorescence of cells that migrated through the filter into L-15 represents random migration whereas the fluorescence of cells migrating into the wells containing the activated serum represents directed migration. The percentage of cells that transverse the membrane in response to the activated serum is determined by the equation:

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\text{Percent increased migration} = \left(\frac{\text{Fluorescence of directed migration}}{\text{Fluorescence of total migration}}\right) \times 100
\]
ing cells - Fluorescence of randomly migrating cells) / (Total cell fluorescence)) × 100.

**Phagocytosis assay**

The ability of phagocytes to take up foreign particles has been used extensively in the study of fish health. Two assays are described here—a chambered microscope slide assay and a cytometric assay. The chambered microscope slide phagocytosis assay was described in Alcorn et al. (2002). Briefly, each leukocyte preparation is placed in triplicate wells of an 8-chambered microscope slide. The slide is incubated for 2 hours in a humid chamber. The non-adherent cells are removed by rinsing each well with cell culture medium. The wells then receive fluorescein (FITC)-labeled *S. aureus* and the slide is incubated for 1 hour in a humid chamber. The wells are again rinsed with cell culture medium to remove the non-phagocytized bacteria. The cells are exposed to a fixative for 30 minutes. The fixative is poured off and the plastic chambers and silicon gasket are removed. After the slide has air dried, a mounting medium containing a fluorescent nuclear stain is placed on each chamber location and a cover slip is placed on the slide. The cells are examined by fluorescence microscopy with concomitant low-light phase contrast microscopy. One hundred leucocytes categorized as macrophages (glass-adherent cells with U-shaped nuclei) are counted and the percentage of phagocytic macrophages (cells with 2 or more FITC-*S. aureus* within their periphery) is determined as shown in Figure 5.

The second assay for phagocytosis uses a flow cytometer (Figure 6) and was adapted from a method described by Li et al (2004). An aliquot of the phagocytic cell preparation is placed in a round-bottom test-tube. Fluorescein-labeled latex microspheres are then added and the tube is incubated for 18 hours to allow the cells to phagocytose the microspheres. Phagocytosis is stopped by the addition of ice-cold fixative. The cell/bead suspension is then layered over a solution having a density greater than the microspheres but less than the cells. The tubes are then centrifuged and the entire supernatant is discarded. This removes any microspheres that were not taken up by the leucocytes. The cell pellet is re-suspended in fixative for cytometric analysis.

In a flow cytometer, the cells are individually passed down a stream of saline solution. As the cells move down this stream they pass through a beam of laser light. The laser light is scattered by the cell, and the degree of scattering is determined by the size and internal complexity of the cell. Macrophages tend to be larger and have greater internal granularity than lymphocytes, so they scatter more laser light.

Several photodetectors measure how this light is scattered. The electrical pulses of the photodetectors can be plotted so that different cell types are represented in different areas of the plot so that only the results of the cell type that falls within the area of interest will be analyzed. Thus, the macrophages can be gated so our results are for just that cell type. The photodetectors can also measure the strength of fluorescent signal that is associated with each cell. The fluorescent signals of 10,000-gated macrophages are collected and plotted on a single parameter histogram. Macrophages with no microspheres will have no fluorescent signal while cells with one or more microspheres will have a fluorescent signal of at least 100 light units. The percentage of macrophages that ingested microspheres is determined using the number of events with a fluorescent signal greater than 100 and the total number of events measured.
Chemiluminescence assay

The production of bactericidal reactive oxygen species by phagocytic cells can be measured by a chemiluminescence (CL) assay. The method described by Scott and Klesius (1981) was modified for salmonids and hybrid striped bass. Each phagocytic cell preparation is diluted in a balanced salt solution containing a compound that releases light when oxidized. Aliquots of each phagocytic cell preparation are placed in multiple wells of an opaque white 96-well microplate.

Salmonid cells will produce a CL response when they are warmed from being on ice to the original fish rearing temperature (FRT). Therefore, while incubating the microplate at the FRT, the chemiluminescent response of the cells is measured for 3 hours (basal CL response) (Figure 7). At the end of the basal CL response period, a compound that causes the cells to release all of their oxidizing molecules (PMA) is added to half of the wells. The amount of light produced by the leucocytes exposed to PMA (PMA response) and the untreated wells are measured for 1 hour. For each well, multiple CL measurements are used to generate a curve representing the kinetic response. An integral calculation is then used to calculate the area under the curve for each well. After subtraction of the untreated CL response from the PMA CL response, the basal CL response and PMA response values are used for statistical comparisons of anterior kidney cell preparations.

Summary

Healthy fish are required for optimal aquaculture production. In addition to good nutrition and adequate water quality, fish need to be protected against infectious disease. The health of fish is maintained by the immune system, which is susceptible to a variety of stressors in the aquaculture environment. Thus, measurements of the humoral and cellular arms of both the innate and specific immune systems of fish can provide information regarding the response of fish to pathogens, to vaccination or to effects of various fish husbandry practices. In addition to serving as research tools, these assays can provide a good indication of overall disease resistance in a population and this information can be used by fish culturists to optimize rearing conditions and increase the health of the stock.

References


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Figure 7. A luminometer can be used to measure chemiluminescence of a sample.
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