Winter School on

# 'RECENT ADVANCES IN DIAGNOSIS AND MANAGEMENT OF DISEASES IN MARICULTURE'

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## Course Manual

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# USE OF IMMUNOSTIMULANTS IN AQUACULTURE MANAGEMENT

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Immunostimulant may be defined as an agent, which stimulate the non-specific immune mechanism when given alone, or the specific immune mechanism when with an antigen. Immunostimulants activate the immune system of animals and render them more resistant to infections by viruses, bacteria, fungi and parasites. They may also be active against human cancer because they activate white blood cells, which recognize and destroy tumor cells. Immunostimulants are valuable for the control of fish diseases.

**Diversity of immunostimulants**

A variety of substances have been shown to have immunostimulatory effects. These substances are microbial derivatives, plant or animal extracts, vitamins and hormones and synthetic chemicals. These immunostimulants mainly facilitate the function of phagocytic cells and increase their antibacterial activities. Several immunostimulants also stimulate the natural killer cells, complement, lysozyme and antibody responses of fish.

**Common Immunostimulants used in aqua culture**

Immunostimulants that have been examined most frequently for their ability to increase the non-specific immune responses of aquatic animals include glucans, and the synthetic drug levamisole. Additionally, animal derived products, such as chitin and abalone extract; bacterial derived products, such as MDP; and plant derived alginates, such as K-carrageen and Spirulina have been examined.

**Fungal Derivatives**

**Glucans**

The immunostimulants present in the cell walls of mushroom and yeasts are mainly β glucans. Glucans appear to show the most promise of all immunostimulants so far examined in fish and shrimp. β glucans are poly glucose molecules linked through β-1,3 bonds in a long chain and with β-1,6 branches consisting of single glucose molecule or chains of glucose molecules. Such glucans can exist in various structural forms and may be in the form of water-soluble oligomers, water soluble or insoluble macromolecules, or particulates.

Glucans extracted from *Saccharomyces cerevisiae* (baker's yeast) is one such type, and is an important structural element of the yeast cell wall. The structures of yeast glucans are polysaccharides composed of smaller units linked together by β- 1,3 bonds. These β -1,3 bonds hold the glucan molecule together; hence the name, β-1,3 glucan. The β-1,3 glucan layer is covered by a mannose-rich protein and lipids. That layer has to be removed to expose the immunostimulatory yeast β-1,3 glucan structures.

**Mode of Action**

In late 1980's Dr. Joyce Czop, at Harvard University, described the mode of action of β-1,3 glucan in stimulating the immune system. There is a specific receptor for β-1,3 glucan on the surface of certain cells, called macrophages that when activated, stimulate a cascade of events turning the body into " an arsenal of defense". There are well defined receptors for β-1,3 glucans on the macrophages of humans and other warm blooded animals, fish and shrimp. The β-1,3 glucan receptor on macrophages is highly specific for the β-1,3 glucan structure as the uptake of β-glucan particles in human or fish macrophages is inhibited only by oligopolysaccharides with the β-1,3 glucan backbone, not by other carbohydrates. The β-1,3 glucan receptor on mcrophages "recognizes" a β -1,3 glucan chain with more than 3 to 5 glucose units. Despite the fact that such small β-1,3 glucan fragments stick to the specific receptor, they are unable to activate the macrophage. The reason for this has not been determined. There is now evidence to show that glucan is, from an evolutionary point of view, the most widely and most commonly observed macrophage activator in nature. β-1,3 glucan has been proven to both stimulate and activate macrophage cells, which can overcome the negative effects of immunosuppression. Activation of macrophages, expressing increased non-specific phagocytic activity allowing macrophages to destroy pathogens more efficiently, frequently preventing disease. The phenol oxidase system is an important element of the disease resistance of crustaceans. It is, however, of crucial biological significance that this latent defense apparatus is able to identify a real infection and not be switched on by signals other than those unique to pathogens. Crustaceans use LPS and the β-1,3 glucan structure as specific signals to activate the prophenol oxidase system. The blood of crustacean contains protein that specifically binds to β -1,3 glucans. When this protein has reacted with β-1,3 glucan, it can bind to a specific receptor on the hemocytes and induce degranulation and release of the prophenol oxidase, which can be converted from its proform into an active enzyme by bacterial LPS or β-1,3 glucans. This specific β-1,3 glucan binding protein, whose structure has revealed, can also act as *opsonin* that stimulates phagocytosis.

**Commercially Available Forms of Glucan**

**Lentinan, Schizophyllan and Scleroglucan**

These substances are soluble glucose polymers with β -1,3 linked backbone and with branches of single glucose molecules attached through β -1,3 linkages. They all form triple helix structure.

**Lentinan**

Lentinan is a polysaccharide extracted from the basidomycete *Lentinus edodes*, the Shiitake mushroom and chemically it is (1,6) branched (1,3)- β-glucan. With two glucose branches for every five β-1,3 glucosyl units in the backbone. Lentinan has also been shown increase disease resistance in fish, for example, injection of 2 to 10 mg/Kg body weight in Carp (Cyprinus *carpio*) increased phagocytic activity of kidney leukocytes and a simultaneous resistance to the bacterial pathogen *E. tarda*.

**Schizophyllan**

This product is extracted from the fermentation broth of the basidomycete fungus *Schizophyllum commune*. The structure of Schyzophyllan is apparently similar to lentinan, but it has one glucose branch for every third glucose in the β-1,3 back bone.

**Scleroglucan**

The β-1,3 glucan is excreted by the basidomycete *Sclerotium glucanum*. Like schizophyllan, scleroglucan has β-1,6 branches at every third glucose in the β-1,3 backbone. It has been demonstrated that scleroglucan induces increased disease resistance and the same cellular effects as schyzophyllan in carp and yellow tail.

**SSG**

This is a highly branched β -1,3 glucan preparation obtained from the culture fluid of the fungus *Sclerotina sclerotiorum*. SSG has been shown to have an immunostimulatory effect and augments macrophage functions *in vivo*. It also acts as an anticancer agent.

**Algal derivative**

Laminaran is a β (1,6)- branched β (1,3)-D- glucan, a major component in sublittoral brown algae, eg: Phaeophyceae,. Almost all β-1,3 )-D- glucan display poor water solubility which makes them less easy to handle than aqueous soluble laminaran. Laminaran obtained from *Laminaria hyperborea* has immunomodulatory effects on anterior kidney macrophages *in vitro* after intra venous adminisration. Intraperitoneal injection of laminaran has also been shown to be preventing mortality caused by *Aeromonas hydrophila* infection in blue gourami, *Trichogaster trichopterus*.

**Synthetic Compound**

**Muramyl peptides**

Muramyl peptides are the molecular entities responsible for the immunostimulatory activity of mycobacterial cell wall preparations of FCA. The minimal structure required for such ativity is N- acetyl muramyl-L-alanyl-D-isoglutamine(MDP)..MDP affects T and B lymphocytes directly, and stimulates macrophages . Various chemical derivatives of MDP have been synthesized to modify their lipid solubility and biological activity . These novel products have been tested mainly for their antitumor activity in humans and as adjuvants in vaccines. It has been shown that MDP potentiate the activity of antibiotics against bacterial infections.

**Levamisole**

The chemical name of this synthetic compound is 2,3,5,6- tetrahydro-6-phenylmedazo[2,1-b] thiazole. Levamisole is an antihelminthic used for the treatment of nematode infections in man and animals. Levamisole in mammals enhance the metabolic and phagocytic activities of neutrophils, and increase the number of phagocytes and leukocytes and level of lysozyme in serum. It has been shown that levamisole also act as an immunostimulant *in vitro* in trout cells and by increasing disease resistance and growth of carp.

**F K - 565**

F K 565 heptanoyl-Y D- glutamyl – (L) meso- diamininopimelyl-(D) alanine is a peptide related to lactyl tetrapeptide (F K- 156) isolated from cultures of *Streptomyces olivaceogriseus* and has been shown to be active against microbial infection in mice. Injection of FK – 565 to rainbow trout increased their resistance to *Aeromonas salmonicida* following the activation of phagocytic cells.

**Animal and plant extracts**

**Chitin and chitosan**

Chitin is a polysaccharide forming the principal component of Crustacean and insect exoskeleton and the cell walls of certain fungi. Rainbow trout injected with chitin showed stimulated macrophage activities and an increased resistance to *V. angullarum* infection. Yellow tail injected with chitin alone also showed increased protection against *P. piscida* challenge, which continued until 45 days after treatment.

**Glycyrrhizin**

Glycyrrhizin is a glycosylated saponin, containing one molecule of glycyrretinine acid, which has anti-inflammatory activities and anti-tumor activities, mediated by its immunomodulatory activities.

**Vitamins**

Vitamins are organic nutrients that are required in small quantities for a variety of biochemical functions and which generally cannot be synthesized by the body and must therefore be supplied by the diet of the various vitamins, Vit C and Vit E are the most important nutrients that influence immune system.

**Vitamin C**

Vitamin C is a water-soluble antioxidant that maintains many metal co-factors in the reduced state. Ascorbic acid deficiency increases disease susceptibility in different species such as rainbow trout and Atlantic salmon and affects the immune system. Several effects of vitamin C deficiencies have been reported, such as reduction of antibody production, and complement activity in Atlantic salmon or reduction of macrophage in channel catfish.

**Vitamin E**

Vitamin E (Tocopherol) is a naturally occurring antioxidant. It is essential for normal reproduction in many animals, hence known as antisterility vitamin. Vitamin E also enhances both humoral and cellular defenses in mammals.

**Hormones, cytokines and others**

**Hormones**

The relationship between neuroendocrine regulation and the immune system has recently become the subject of intense investigation. It is also known that growth hormone (GH) and prolactin (PRL) directly affect immune competent cells (macrophages, lymphocytes and NK cells). In fish exogenous GH has mitogenic activity on lymphocytes and activate NK cells.

Melanin stimulating hormone (MSH) and melanin concentrating hormone (MCH) stimulate phagocytosis by head kidney leucocytes of rainbow trout is vitro.

Ainsworth *et al* (1994) showed that oral administration of Vita stim taito at concentration of 0.1 percentage in the feed led to more efficient antibody production in cat fish against live *Edwardsiella ictaturi*. The effect depended on the dose of Vita stim taito in the feed and on the size of the feed. Juvenile rainbow trout (0.12g) fed rations contains 0.2 aand 2 mg/Kg peptidoglycan and challenged the fish after 28 and 56 d ay by

immersion in suspension of *V. anguillarum*. The peptidoglycan did not affect fish growth significantly, although a slight growth enhancement was recorded after 56 d at 2 mg/Kg. The fish were markedly more resistant to bacterial challenge after peptidoglycan administration fro 28d at both concentarion pf the immunostimulants, but only the low dose (0.2mg/Kg) produced increased disease resistance when challenged after 56d.

**The Dose / Response Relationship**

Immunostimulants does not show a linear dose /effect relationship, instead they most often show a distinct maximum at a certain intermediate concentration and even a complete absence of effect or toxicity, at high concentration. The explanations for these phenomena are still speculative and include competition for receptors (analogous to substrate inhibition of enzyme), over stimulation resulting in exhaustion and fatigue of the immune system..

Kajita *et al* (1990) showed that chemiluminiscent effects of phagocytic cells in rainbow trout were increased by injection of levamisole at 0.1 and 0.5 mg/Kg. However, they also reported that the injection of 5 mg/Kg of levamisole did not produce this immunostimulant effect. Robertsen *et al* ( 1944) reported that the increase in respiratory burst activity of glucan treated macrophages was maximal at glucan concentrations of 0.1-1 mg/ml, where as at 10 mg/ ml no effect was seen and at 50 mg/ml glucan was inhibitory.