Immunostimulants, Vaccines, and Environmental Stressors in Aquaculture: NBT Assays to show Neutrophil Activity by these Immunomodulators

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Abstract

While neutrophil activity assays have been used in human and veterinary medicine for diagnosis of blood neutrophil abnormalities, they are rarely used because of difficulties of handling nitro blue tetrazolium (NBT) solutions, drawing and preparation of blood samples and subjectivities in visual readings. We have developed modifications for a simplified NBT assay that can be used in the diagnosis and tracing the effectiveness of immunostimulants, vaccines and environmental stressors of fish. While training and practice for biologists and technicians is needed, the NBT assays can yield good results when applied to fish.

Introduction

Tracing induced changes in fish by the use of immunostimulants, vaccines or environmental stressors (such as aquatic pollutants) is an important part of present day aquaculture (Anderson 1992). Some of the main immunostimulants used are levamisole (Siwicki et al 1990, glucans (Jeney and Anderson 1993), chitins (Cuesta et al 2003), and many others. It is well known that these agents stimulate the nonspecific immune response (also called innate or natural protection) and boost the specific immune response. Therefore, immunostimulants can also be used as adjuvants, to heighten the specific immune response. While Freund's adjuvants are effective in boosting the fishes' immune response, these formulae cannot be used in food fish because of scarring or tissue injury. As a result, low molecular weight oils are now being mixed and tested with some bacterins for fish bound for the marketplace. The presence of pollutants and contaminants in the environment can reduce the nonspecific and specific immune responses in fish. In the field, the final (in vivo) results of exposure to these agents of fish are evident by protection against diseases, or morbidities or mortalities. The NBT in

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vitro assays can give early indications or predictions concerning these end results.

The pathways of the nonspecific and specific immune response after vaccination are well described in fish and follow the patterns of other animals. Salmonids, being short-lived species, and living in cooler temperatures that limit rapid physiological pathways may depend more upon the nonspecific response for protection against diseases. These pathways are less defined and the points of action of different immunostimulants which may be particulate, soluble or globular have individual characteristics. Many recent experiments have shown the immunostimulants can be given alone to induce the in vitro and in vivo responses (Juina and Wu. 2004).

Assays for detecting whether or not the administrations of the immunostimulants or vaccines are effective have been difficult to develop. In our case, working with the specific strain of Yersinia ruckeri we can follow the development of serum antibody (ABY) and antibody producing cells (APC - also designated PFC - plaque-forming cells) after injection and bath. While the commercial vaccines used in the field usually do not yield easily to the initiation and detection of ABY or APC, protection against the diseases in evident under many conditions. It is frustrating to the field biologist that quick ABY or APC assays are not available for the confirmation of a commercial vaccine‘s effectiveness. We know from the research laboratories that ABY and APC occur when antigens such as Bovine Serum Albumin, Sheep Red Blood Cells or Yersinia 0-antigens are used in fish. The reasons for the difficulty in diagnosis of the specific immune response for other vaccines may include: 1) Antigens in the vaccine are different from those used for the diagnostic assays. 2) A small (undetectable) amount of antibody is produced in commercial situations and therefore not detectable by our less sensitive assays. 3) The vaccine stimulates protection mechanisms such as macrophage activation that present assays cannot detect.

The case is even more difficult when only the immunostimulants are used, as these

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substances do not induce ABY or PFC. Therefore, we have developed assays derived from human and veterinary medicine for the assays of the effectiveness of immunostimulants. However, for cold-blooded animals such as fish, certain modifications have to be made such as in timing and temperatures of incubation. The NBT slide tests have proved to give the most accurate results for us.

The detrimental effects of natural environmental stressors such the presence of heavy metals or man-derived aquatic pollutants are well recognized by morbidity factors such as lesions and tissue abnormalities. The NBT assays can also give early indicators of the pollutants as to how they are affecting the hemopoietic systems of the fish

Materials and Methods

Our standard method with salmonids and similar fishes is to first obtain the blood sample 3-5 days after immunostimulation, vaccination or pollutant exposure either by a direct caudal cut or by syringe. Two drops of blood are immediately placed in separate spots on a clean, glass slide and incubated on wet paper towels for 30 minutes in a covered, humid chamber at room temperature.

After incubation, the drops of blood are gently washed with a saline solution (0.85% NaCl) from a Pasteur pipette, and a drop of the NBT solution is placed on top of the remaining (glass-adherent) blood cells and overlaid with a square cover slip. The slides are incubated for another 30 minutes, and then read through the microscope. Longer or shorter incubations times can be modified for individual users or conditions. Blue-staining cells are counted and numbers compared between test and control groups of fish.

Preparation of the NBT solution: We use NBT, reagent grade, obtained from Sigma Co. MI, USA, at a 0.2% in a saline solution. Other brands or concentrations may apply in particular situations. There may be some difficulty in bringing the chemical into

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solution, In such cases, the NBT is first placed into distilled water, and slowly heated, without boiling, if possible. Then the appropriate amount of NaCl is slowly added. There should be no precipitate in the solution. Solutions are best if prepared fresh for each series of tests. NBT solutions can be distributed in small aliquots and frozen for later use.

This simple NBT test can be used for showing the neutrophil stimulation after fish have been subjected to an immunostimulant, vaccine, and environmental stressors. This indicator of change in neutrophils is an important addition to other parameters that show physiological changes, such as hematocrits, leukocrits, blood cell counts -- the more correlations to other indicators, the better.

Results and Discussion

Immunostimulants:

Glucans and other immunostimulants are now being added to fish feeds and vaccines. In some cases they are injected alone at points of time near predictable fish stressors. We have demonstrated the effectiveness of these agents in reducing mortalities. While many immunostimulants are effective, the glucans are the most commonly used at this time.

The NBT assays can be used to test the effect of the immunostimulants. After standard kinetic curves have been set up for individual environments and species, the effects of different doses and preparations can be tested. For salmonids, the numbers of blue-NBT staining neutrophils peak, usually three days after injection of glucan. For instance, an average slide viewing on a 10X objective may reveal 10-30 cells from a control fish in contrast to 100-200 cells in a treated fish. Research has shown that it is important to sample as many fish as possible because occasionally control fish may show a high number of cells and a treated fish may likewise show low numbers. If any gross physical

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abnormalities are noted before sampling, the data from that fish should be disregarded.

It will be interesting to follow research concerning glucan use in fish, as glucan receptors are being described on fish cells. Eventually the cell receptors will be more defined as has already been done in some cases (Romo-Fugueroa et al. 2004). The activity of the neutrophils in the NBT test depends upon two factors: glass adherence, and the production of oxidative radicals. It will be interesting to know whether there is some relationship between these activities and the presence or expansion of the numbers of glucan receptors.

Vaccines:

Active immunization is routinely used in aquaculture against vibriosis, furunculous, yersiniosis, and bacterial kidney disease. When these vaccines are injected, immunostimulants can be easily added to heighten the immune response.

When testing vaccines, usually dosage studies are done to determine the lowest doses of a vaccine that can be given to induce antibody production and protection from disease. The NBT tests also follow these dosage patterns. The higher the doses of vaccine, the more NBT-staining cells induced. Oral and bath administration of vaccines may show slightly different time patterns. Since bacterial endotoxins are active inducers of the innate immune response, vaccines containing these materials are often excellent stimulators of NBT-staining cells.

We have used this assay to show the effectiveness of Yersina ruckeri bacterins in a specific immune response -- later doing ABY and PFC assays. The major advantage of the NBT test under this application is that it can be done 3 days after vaccination, whereas the earliest for the appearance of PFC is 7 days and circulatory ABY appears in 10-14 days (Figure 1). And, as noted, sometimes this is the only in vitro evidence of the

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vaccines effectiveness.

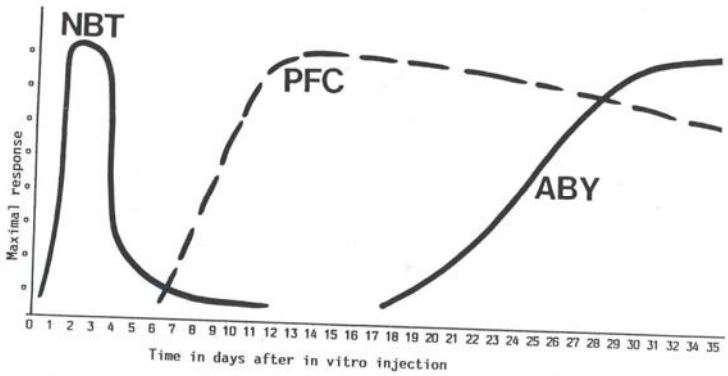

Figure 1. The kinetic patterns of the NBT-staining cells appearing in the blood of fish injected with a vaccine (Yersinia ruckeri 0-antigen) precedes the appearance of Plaque-forming cells (PFC) in the spleen and the appearance of serum antibody (ABY). In these tests, rainbow trout were injected once and held in 10C water. Kinetic patterns may differ for other environments and fish species.

Environmental Stressors:

Pollutants and contaminants in aquatic environments can affect blood cell parameters. The NBT test shows that numbers of neutrophils are influenced by the presence of unhealthy materials in the environment. This is most obvious when fish are showing physical signs, such as small skin lesions. Some morbid fish from adverse environments may show no NBT-staining cells. When sampling fish for environmental effects, it may be important to adjunct the tests carefully with hematocrit samples, blood cell counts and, if possible, leukocyte differential counts. Laboratory tests can easily show the reduction of NBT-staining cells when fish are treated with high concentrations of pollutants. Under natural conditions, where the actual pollutant may not be known, general conclusions can be drawn about the health of the fish.

We have used these assays to show that if fish are exposed to aquatic phenol concentrations immediately before bath immunization, the resultant immune response is less, demonstrating that the uptake of these antigens by bath can be blocked. The

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contaminant exposure affects the innate immune response as well and the active, antibody production (Figure 2).

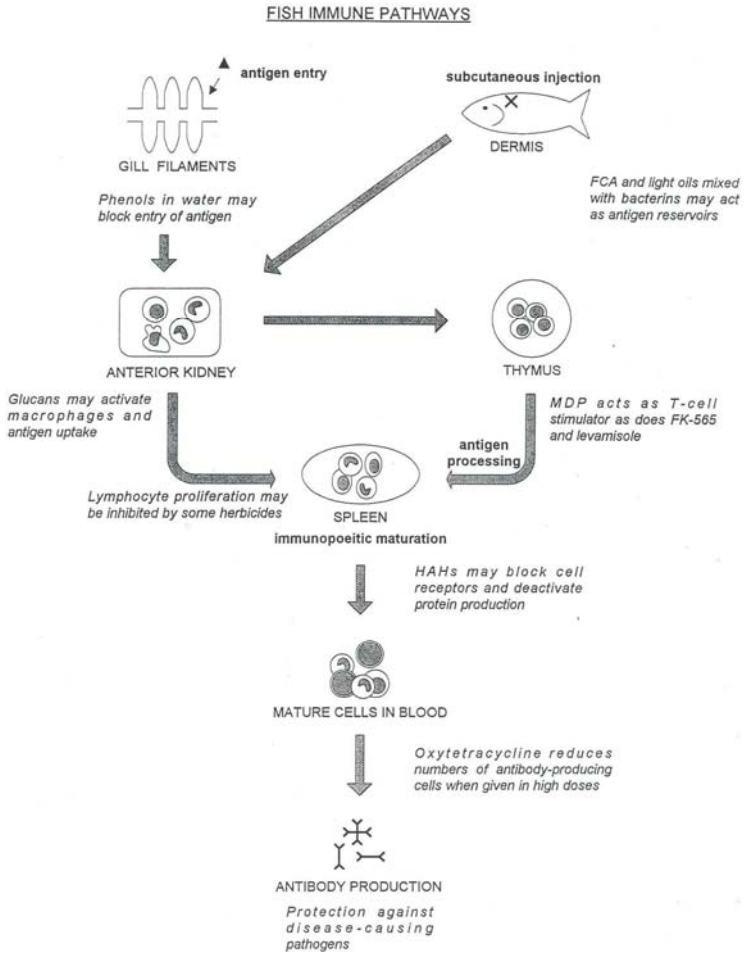

Figure 2. This schematic diagram shows antibody production in rainbow trout can be affected by different influencing factors. For instance, the presence of phenol in the water may reduce or block the uptake of antigen in bath immunization. In contrast, glucan added to a vaccine may act as an adjuvant, further stimulating the immune response. NBT tests can be used to give preliminary evidence of vaccine effectiveness.

Reservations and modifications may be necessary:

A main difficulty is in reading the assays. Practice is needed in ensuring the neutrophils as glass-adherent cells are sticking to the glass in sufficient numbers. Fish Biologists and technicians need practice and training in proper drawing of blood, assay procedures, and

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doing final readings. With the NBT assays the action of immunostimulants, vaccines and environmental stressors can be surveyed, and eventually with other factors, the mechanisms of the nonspecific and specific immune response that are affected will be known (Figure 2).

Other difficulties include ensuring that the control fish have not been held under some situation of stress. We usually use two control groups when testing an immunostimulant by injection. For instance, one control group is given a sterile injection of saline; another control group is carefully held in a separate tank and given no stimulants. Often the results show that the latter have very few NBT cells; the saline-injected have more; and the test group, given the immunostimulant, a high number.

We have developed many modifications of the NBT assays, using spectrophotometer readings and fluorescence. Indeed some of these methods are very good. The drawbacks include factors such as that more expensive equipment is needed as well as highly trained and observant personnel. In addition, using this equipment often takes more time. These simplified tests can be easily performed in the field. Further refinements are developing, but usually require additional equipment and need a more complete laboratory available.

References

Anderson, D.P. 1992. Immunostimulants, adjuvants, and vaccine carriers in fish: Applications to aquaculture. Annual Review of Fish Diseases 21, 281-307.

Anderson, D.P., T. Moritomo, R. de Grooth. 1992b. Neutrophil, glass-adherent, nitro blue tetrazolium assay gives early indication of immunization effectiveness in rainbow trout. Veterinary Immunopathology 30, 419-429.

Cuesta, A., M. Angeles Estaban, and J. Meseguer. 2003. In vitro effect of chitin particles on the innate cellular immune system of guilthead sea bream (*Sparus aurata L.*). Fish and Shellfish Immunology 15, 1-11.

Jian, J., and A. Wu. 2004. Influences of traditional Chinese medicine on non-specific immunity of Jian carp (*Cyprinus carpio var. Jian*). Fish and Shellfish Immunology 16, 185-191.

Jeney, G., and D.P. Anderson. 1993. Glucan injection or bath exposure given alone or in combination with a bacterin enhance the nonspecific defence mechanisms in rainbow trout (*Oncorhynchus mykiss*) Aquaculture 116, 315-329.

Jimenez-Vega, F., R.R. Sotelo-Mundo, F. Ascencio, and F. Vargas-Albores. 2002. 2-3-beta-D glucan binding protein (BCBP) from the white shrimp, *Penaeus vannamei*, is also a heparin binding protein. Fish and Shellfish Immunology 13, 171-181.

Romo-Figueroa, M.G., C. Vargas-Requena, R.r. Sotelo-Mundo, F. Vargas-Albores, I. Higuera-Ciapara, K. Soderhall, and G. Yepiz-Plascencia. 2004. Molecular cloning of a beta-glucan pattern-recognition lipoprotein from the white shrimp, Penaeus (*Litopenaeus) vannamei*: correlations between the deduced amino acid sequence and the native protein structure. Developmental and Comparative Immunology 28, 713-726.

Siwicki, A.K., D.P. Anderson, and O.W. Dixon. 1990. In Vitro immunostimulation of rainbow trout (*Oncorhynchus mykiss*) spleen cells with levamisole. Developmental and Comparative Immunology 14:2. 231-237.

Zapata, A.G., M. Torroba, F. Alvarez, D.P. Anderson, O.W. Dixon, and M. Wisniewski. 1987. Electron microscopic examination of antigen uptake by salmonid gill cells after the immunization with a bacterin. Journal of Fish Biology 31 Suppl. A:209-217.

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