INVESTIGATIONS INTO IMMUNOSTIMULANT USE FOR THE ENHANCEMENT OF IMMUNITY TO THE FURUNCULOSIS BACTERIUM, *AEROMONAS SALMONICIDA*, IN COHO AND CHINOOK SALMON.

by

Lee H. Nikl

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APPROVAL

Name: **LEE H. NIKL**

Degree: **Master of Science**

Title of Thesis:

INVESTIGATIONS INTO THE USE OF IMMUNOSTIMULANTS FOR THE ENHANCEMENT OF IMMUNITY TO THE FURUNCULOSIS BACTERIUM, *AEROMONAS SALMONICIDA* IN THE COHO AND CHINOOK

Examining Committee:

Chairman: Dr. John M. Webster

Dr. Lawrence J. Albright, Senior Supervisor, Dept. Biological Sciences, SFU

Dr. Brian A. McKeown, Professor, Dept. Biological Sciences, SFU

Dr. Trevor P.T. Evelyn, Adjunct Professor, Resident Scientist, DFO, Pacific Biological Station, Nanaimo, B.C.

Dr. Michael Kent, Fish Health Research Scientist, DFO, Pacific Biological Station, Nanaimo, B.C., Public Examiner

Date Approved: 25 Sept., 1991.

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Investigations into the use of immunostimulants for the enhancement of immunity to the furunculosis bacterium *Aeromonas salmonicida* in coho and chinook salm

Author:

(signature)

Lee Nikl

(name)

October 15, 1991

(date)

ABSTRACT

Seven substances were tested for their ability to enhance immunity in juvenile coho (*Oncorhynchus kisutch*) and juvenile chinook (*Oncorhynchus tshawytscha*) salmon to *Aeromonas salmonicida*, the causative bacterium of furunculosis. The screening trials involved injection of the test substance into fish, along with a bacterin consisting of formalin-killed *A. salmonicida* cells (=*A. salmonicida* bacterin). After 21 days, these fish were challenged with live *A. salmonicida* cells and survival in this group was compared to the survival in a control group treated with bacterin only. Consistent and significant protection was observed in the groups receiving purified and highly depolymerized VitaStim-Taito (VSTp), lentinan, and formalin-killed *Renibacterium salmoninarum* cells in combination with *A. salmonicida* antigen. However, because of considerations such as cost and ease of production, it was decided to restrict further study to only one of these -- VitaStim-Taito (VST).

In further tests, VSTp (20 mg·kg^{-1} fish) proved effective at enhancing immunity to *A. salmonicida* when injected without bacterin, with the protection lasting for at least 28 days following a single intraperitoneal injection. However, when administered by immersion, VST was found to be ineffective. Two forms of VST were used in the immersion trials: VSTp (100 ppm bath) and a crude form of VST (150 ppm bath). Both of these products were tested on their own and in combination with an immersion-delivered *A. salmonicida* bacterin.

The practicalities of commercial aquaculture production facilities make injection vaccination an unsuitable method for the large-scale immunization of fish. Therefore, VST's effects were examined when administered orally. Promising results were obtained with crude VST mixed into a steam-pelleted,

dry, commercial-type diet at concentrations of either 0.1 or 1.0 %. Groups of juvenile chinook salmon receiving a 2 week regime of food containing VST were significantly protected from challenge by *A. salmonicida* but not when challenged by a highly virulent strain of *Vibrio anguillarum*. The results with *A. salmonicida* are encouraging because oral administration would prove practical on a commercial fish farm.

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TABLE OF CONTENTS

APPROVAL ii

ABSTRACT iii

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF TABLES viii

LIST OF FIGURES x

INTRODUCTION 1

- Furunculosis of Fish 2
- Virulence Mechanisms of *A. salmonicida* 3
- The Immune System of Fish 5
- Furunculosis Vaccination 7
- Immunostimulant Use in Fish 9

MATERIALS AND METHODS 11

- Experimental Animals 11
- Maintenance of *A. salmonicida* Virulence 12
  - Autoaggregation 13
  - Coomasie Dye Test 14
- Challenge Procedure 15
  - Immersion Challenge 15
  - Cohabitation Challenge 16
  - Statistical Analysis 17
- Efficacy Trials With Seven Candidate Immunostimulatory Substances 17
  - Immunostimulants 19
  - Antigen Preparation 20

Influence of VST, Lentinan, and *Renibacterium salmoninarum* Bacterin on the anti-*Aeromonas salmonicida* Agglutinin Response........22
Blood Collection........22
Antigen Preparation........23
Antibody Titre........23
The Influence of VitaStim-Taito on the Nonspecific Immune Response of Juvenile Coho Salmon........25
Assessment of the Efficacy of Immersion Administered VitaStim-Taito With and Without Antigen........26
Assessment of the Prophylactic Efficacy of VitaStim-Taito when Administered via the Feed........27
Diet Preparation........27
Experimental Design - Oral Administration of VitaStim-Taito........28
RESULTS........33
Efficacy Trials With Seven Candidate Immunostimulatory Substances........33
Effect of Selected Immunostimulants on the Agglutinin Response to *A. salmonicida* Antigen........35
Stimulation of Nonspecific Immunity by VitaStim-Taito........37
Immersion Administration of VitaStim-Taito........40
Oral Administration of VitaStim-Taito........42
DISCUSSION........47
SUMMARY........55
REFERENCES........56

LIST OF TABLES

Table 1. Immunostimulants used for the initial screening study.................... 21

Table 2. Composition of the basal diet used in the oral administration trials.. 30

Table 3. Composition of vitamin supplement premix used for VST feed study...31

Table 4. Composition of mineral supplement premix used for VST feed study...32

Table 5. Results of screening of various immunostimulants for their ability to potentiate the immune response to *A. salmonicida* bacterin.. 34

Table 6. Influence of the immunostimulants VitaStim-Taito (VST), lentinan, and *R. salmoninarum* bacterin (RSB) on the agglutinin response to an *A. salmonicida* bacterin........................ 36

Table 7. Influence of VitaStim-Taito in crude (VSTm) and pure form (VSTp) administered by immersion with and without *Aeromonas salmonicida* bacterin (Ag) on survival following immersion challenge with *A. salmonicida*...41

Table 8. Heterogeneity testing of replicate mortality data obtained in the oral administration of VitaStim-Taito (VST) trials (*Aeromonas salmonicida* challenge)..43

Table 9. Heterogeneity testing of replicate mortality data obtained in the oral administration of VitaStim-Taito (VST) trials (*Vibrio anguillarum* challenge).. 44

Table 10. Survival of juvenile chinook salmon (*Oncorhynchus tshawytscha*) fed diets containing various amounts of

VitaStim-Taito (VST) and immersion-challenged with *Aeromonas salmonicida*.......... 45

Table 11. Survival of juvenile chinook salmon (*Oncorhynchus tshawytscha*) fed diets containing various amounts of VitaStim-Taito (VST) and immersion-challenged with *Vibrio anguillarum*.......... 46

LIST OF FIGURES

Fig. 1. Mortalities in coho salmon (*Oncorhynchus kisutch*) treated by injection with saline, VST, or *A. salmonicida* bacterin and challenged 20 days later (day 0) with *A. salmonicida*....39

INTRODUCTION

Fish culture has been practiced for centuries in various parts of the world. In British Columbia, fin fish culture has until recently been a government-run operation, the aim being to enhance the wild-stock fisheries. A more recent enterprise in this province has been the commercial net-pen culture of what are predominantly salmonid species.

Many fish farms on this coast have experienced problems with fish diseases such as vibriosis, furunculosis, and bacterial kidney disease (Evelyn and Ketcheson, 1980). Treatment, usually consisting of antibiotics, has been employed with some success against these and other diseases. This approach, however, is expensive, provides only short-term benefit, and risks generating drug resistant strains of bacteria. These shortcomings, combined with a strong popular sentiment against the use of antibiotics in food fish, continue to make the development of effective vaccines for diseases such as furunculosis and bacterial kidney disease highly desirable.

Commercial vaccines for furunculosis are available but their efficacy has not been satisfactory, the protection afforded often being weak (Ellis, 1988). Vaccine development for furunculosis has tended to focus on exploiting the specific immune system. However, this has not been a successful approach in all cases. Other, more primitive components of the immune system, particularly the reticuloendothelial system, have not been fully exploited in fish vaccine development. In the present study, several immunostimulating compounds were evaluated for their ability to enhance a simple, bacterin-type, injectable furunculosis vaccine. One of these agents was then further assessed for its ability to enhance, nonspecifically, the immune response against the furunculosis bacterium when administered by either the immersion or the oral route.

Furunculosis of Fish

Furunculosis is a systemic bacterial infection of fishes. It is caused by a Gram negative, non spore-forming, non-motile, rod-shaped bacterium. *Aeromonas salmonicida*, described by Griffin *et al.*, (1953), remains the cause of one of the most serious diseases of cultured salmonids (Roberts and Shepherd, 1986). The existence of atypical isolates has led to subspecies designations for *A. salmonicida* (Kimura, 1970; McCarthy, 1975).

Early clinical signs of infection by *A. salmonicida* include inappetance and discoloration of the skin. Fish so infected tend to exhibit sluggish behaviour and can often be found at the outlets or the margins of the holding units. Later in the infection, hemorrhagic areas are often observable near the base of the fins and in the area around the anus. In acute furunculosis, grossly observable signs are often not present. In subacute to chronic infections, external lesions or "furuncles" may be conspicuous. The common name given to the disease caused by *A. salmonicida* derives its name from these lesions. Their presence, however, is not considered to be evidence of an *A. salmonicida* infection as other pathogens can produce similar gross clinical signs. Diagnosis of furunculosis is based on isolation of the pathogen from diseased individuals (Post, 1987).

Chemotherapeutic treatment of furunculosis is based on the administration of various antibiotic compounds (sulfamerazine, for example). Aoki *et al.* (1983) indicated that the incidence of *A. salmonicida* resistance to antimicrobial agents is increasing. Furthermore, Grondel *et al.* (1985) demonstrated that some of the antibiotics used to treat furunculosis could lower the mitogenic activity of fish leucocytes. Such a depression of the

immune system could further aggravate the disease problem. Consequently, techniques that prevent the initial establishment of the disease would be highly desirable. Such techniques might include the use of preventative vaccines or compounds capable of enhancing the immune response of fish.

Virulence Mechanisms of *A. salmonicida*

It is useful to be familiar with the mechanisms of virulence of a given pathogen when developing a vaccine or treatment strategy. Virulence factors are considered to be good candidates as constituents of vaccines (Ellis, 1988) because when neutralized by the immune system, the pathogen is put at a disadvantage relative to its host.

The virulence mechanisms of *A. salmonicida* are the subject of considerable controversy. It may be said that as yet, the specific factors responsible for virulence in all strains of *A. salmonicida* are unknown. However, there has been a suggestive association with certain of the extracellular products produced by this bacterium and with the A-layer, a structure associated with the intact cell.

The role played by the extracellular products (ECP) of *A. salmonicida* continues to be unclear (Ellis *et al.*, 1988a). Components isolated from the ECP of this pathogen include proteases (Shieh and McLean, 1975; Ellis *et al.*, 1988b), leucocidal factors (Fuller *et al.*, 1977), and hemolysins (Nomura *et al.*, 1988). The extracellular products (ECP) of *A. salmonicida* have been shown to be capable of producing nearly all of the signs associated with furunculosis (Ellis *et al.*, 1981) and have been shown to be important in the pathogenesis of furunculosis. Some of the apparent confusion regarding the ECP may be at least partially explained by the recent work of Lee and Ellis

(1989). A major lethal exotoxin of *A. salmonicida* was found to be a glycerophospholipid:cholesterol acyltransferase complexed with lipopolysaccharide. This toxin showed hemolytic, leucolytic, and cytotoxic activities, all of which have been previously identified within different fractions of the ECP.

Another virulence factor associated with the intact *A. salmonicida* cell is the A-layer (Udey and Fryer, 1978; Trust *et al.*, 1983). The A-layer is an extracellular protein layer that is believed to be critical to the pathogen's survival within the host (Trust *et al.*, 1983). The A-layer, in conjunction with lipopolysaccharide, is thought to impart resistance to the bactericidal activities of complement and to phagocytosis (Sakai and Kimura, 1985; Munn *et al.*, 1982). However, as with ECP, the reported importance of the A-layer for virulence is also in question (Ellis *et al.*, 1988b).

At present, the relationship between the ECP, virulence, and immunogenicity is not a clear one. Such confusion may be related to the properties of different *A. salmonicida* strains (see Cipriano *et al.*, 1981 for example) and the generalization of observations from a particular study with a limited number of strains to all strains within the species. Identification of a protective antigen, effective for all strains of *A. salmonicida* will be a major undertaking but will no doubt contribute to the efficacy of furunculosis vaccines.

The goal of the present study was to investigate manipulation of the immune system of fish. For this purpose a known strain of *A. salmonicida* (strain 76-30, Olivier *et al.*, 1985a) was selected. While universal markers of virulence for *A. salmonicida* are still unknown, the association between virulence and non-virulence of this specific strain appears to be related to the presence of the A-layer (Olivier *et al.*, 1985a) which is easily assayed for.

Furthermore, the presence of this same protein is also related to immunogenicity (Olivier *et al.*, 1985b) in this strain.

The Immune System of Fish

Discussions of the immune system of higher vertebrates often approach the subject by dividing this system into two components: the humoral component, containing all of the soluble factors with direct anti-pathogen activity, and the cellular component involving cell to cell communication through soluble mediators and cellular effector mechanisms (North, 1978). Cell-mediated immunity in fish is not well characterized. This is probably the result of the current inability to demonstrate the existence of both B and T cells within a group of lymphocytes. While such lymphocyte heterogeneity is well established in many mammals and easily demonstrated through the presence of cell surface antigens, it remains to be conclusively demonstrated in fish. Nevertheless, despite an inability to define the cell types on the basis of surface markers, it would appear that their functional analogues are present in salmonids (Etlinger *et al.*, 1976; Lobb, C.J. and Clem, 1982; Smith and Braun-Nesje, 1982; Irwin and Kaattari, 1986; Secombes, 1987).

A functional property differentiating B lymphocytes and T lymphocytes is their response to mitogens. Lipopolysaccharide (LPS) is known to induce a mitogenic response in B cells but not in T cells, while concanavalin A, on the other hand, selectively stimulates only T cells. Etlinger *et al.* (1976) found that various rainbow trout lymphoid tissues exhibit a localization of different mitogenic responses. Cells extracted from rainbow trout thymus showed a responsiveness to concanavalin A but not to LPS. Conversely, leukocytes

extracted from the anterior kidney showed a strong mitogenic response to LPS but failed to respond to concanavalin A. Cells taken from the peripheral blood and spleen were, as might be expected, responsive to all mitogens tested. It was proposed that in rainbow trout, different populations of lymphocytes exist.

A marker used to distinguish B cells from T cells is the presence of membrane-bound immunoglobulin M (mIgM) on B-cells. Lobb and Clem (1982) showed that only a certain portion of catfish (*Ictalurus punctatus)* lymphocytes responded to mouse monoclonal antibodies against catfish IgM. The researchers used several different monoclonal antibodies directed against catfish IgM and found that only about 40 % of the lymphocytes were positive for surface bound immunoglobulin. It was postulated that two distinct lymphocyte subpopulations, at least one of which had B cell-like characteristics, were present in catfish.

DeLuca *et al.* (1983), using monoclonal anti-trout IgM antibodies, found lymphocyte subpopulations possessing mIgM. Furthermore, when the surface immunoglobulin positive subpopulation was removed from culture, the mitogenic response to LPS, a B cell mitogen, was depleted. The cell culture depleted of mIgM positive cells showed no suppression of a concanavalin A induced mitogenic response. The authors demonstrated the presence of two different subpopulations of lymphocytes. One of these populations had the properties usually associated with B cells and was considered to be B-like. The other population, though not clearly defined, was presumed to be T-like.

Cellular interactions, reminiscent of the types seen in higher vertebrates, are also seen between the T-like cells and macrophages of fish. Smith and Braun-Nesje (1982) demonstrated that concanavalin A-stimulated

lymphocytes produced a soluble factor capable of activating macrophages. The factor released into the culture medium resulted in changes to fish macrophages that were similar to those known to be indicators of activation in mammalian studies (North, 1978). Similar results were observed by Secombes (1987).

It is accepted that salmonids can produce an agglutinin (antibody) response to pathogens such as *A. salmonicida* (Krantz *et al.*, 1964; Spence *et al.*, 1965; Cipriano, 1982a; Cisar and Fryer, 1974; and McCarthy *et al.*, 1983). These antibodies have been characterized in coho salmon (*Oncorhynchus kisutch*) by Cisar and Fryer (1974). Only one class of antibody, or immunoglobulin, is present in fish compared to the five separate classes seen in mammals (Ellis, 1981). The coho salmon immunoglobulin resembles mammalian immunoglobulin M (IgM), but has a tetrameric structure in contrast to the pentameric IgM of mammals (Tizard, 1984).

Non-immunoglobulin serum constituents have more recently become recognized as important anti-pathogen components. For example, the presence of complement has been shown to be influential in the bactericidal activity of serum against *A. salmonicida* (Sakai, 1983). In further *in vivo* studies, Sakai (1984) found that serum mixed with ECP at a 19:1 ratio was able to neutralize the toxicity of ECP, and, when injected into rainbow trout, resulted in no mortality. However, when this serum was decomplemented, the same proportion of serum was unable to neutralize the toxins.

Furunculosis Vaccination

The first furunculosis vaccination attempts with fish involved chloroform-killed *A. salmonicida* cells in an orally-administered vaccine (Duff,

1942). This vaccine was protective for cutthroat trout (*Oncorhynchus clarki*). However, subsequent work with orally administered furunculosis vaccines met with little success (Snieszko and Friddle, 1949; Krantz *et al.* 1964; Hara *et al.* 1976). With the demonstrated effectiveness of sulphonamide drugs (Snieszko and Friddle, 1949), there was a loss of interest in vaccine research until the development of drug resistance in *A. salmonicida* strains (Munro, 1984).

The discouraging results with the furunculosis pathogen may be due in part to its chemical make-up. Although the immunogenic antigen(s) of *A. salmonicida* have yet to be unequivocally identified, several of the proposed immunogenic antigens are proteins (Cipriano, 1982b; McCarthy *et al.*, 1983, Olivier *et al.*, 1985b). Due to the action of digestive acids and enzymes, it is likely that during passage through the digestive system of the salmonid, the immunogenic antigen is destroyed. Intact protein absorption does occur in the posterior portion of the salmonid intestine (Georgopoulou and Vernier, 1986; McLean and Ash, 1987; James *et al.*, 1988; Hart *et al.*, 1988), lending some promise to possible future oral vaccination against furunculosis with a protected antigen. However, this work is beyond the scope of the present study. Oral administration of drugs and biologicals removes the necessity of fish handling -- a practice that can itself result in stress-related suppression of the desired response (Kaattari and Tripp, 1987; Saad, 1988).

Coho salmon as small as 1.2 g were capable of responding to *A. salmonicida* antigen (Paterson and Fryer, 1974). Furthermore, while temperature appeared to affect the length of time taken to respond, fish were capable of responding at all temperatures in the range (6.7 to 17.8 ^{o}C) tested.

Many studies of furunculosis vaccination have often stopped at the point of assessing antibody titre. However, Michel and Faivre (1982) demonstrated that survival during an *A. salmonicida* challenge was independent of the antibody titre. Furthermore, Smith *et al.* (1980) showed that while circulating agglutinins failed to play a dominant role in immune protection, cell mediated immunity (CMI) was associated with immune protection against this specific pathogen. Other investigators (Olivier *et al.*, 1986; Yano *et al.*, 1989) have since found that an exploitation of CMI in fish has proven beneficial in furunculosis prophylaxis.

Despite considerable research on the topic of furunculosis vaccines (Ellis, 1988) heavy losses of cultured salmonids due to furunculosis continue to occur. Chemotherapy is therefore frequently used to control the disease--an approach that is expensive, provides only short term benefit, and risks generating drug-resistant strains of the causative bacterium, *A. salmonicida*. Such shortcomings make the development of an effective furunculosis vaccine highly desirable.

Immunostimulant Use in Fish

Although the development of humoral immune factors specific for *A. salmonicida* is a typical response to exposure to the pathogen, the presence of these factors (antibodies) is not necessarily associated with protection (Michel and Faivre 1982; Cipriano, 1982b and 1983; Olivier et al., 1985b). Smith *et al.*, (1980) showed that immunity to furunculosis was more closely associated with the cellular components of the immune system than with the humoral ones. It is conceivable that furunculosis vaccination

attempts might benefit from vaccines specifically designed to take advantage of the cell-mediated host defences.

One way to design a vaccine that will exploit the cellular immune response is to modify the antigenic determinants so that they preferentially stimulate cellular immunity. This approach was applied to sheep red blood cells (Parish 1972) and to bovine serum albumin by Coon and Hunter (1973). Another approach is to include an immunostimulant in the vaccine (Allison and Byers, 1986; Panangala et al., 1986). By combining an antigen with an immunostimulant, enhanced protection may be obtained, apparently through a more complete exploitation of the host's immune system.

Olivier et al. (1985a) were the first to demonstrate in salmon that the efficacy of an injected *A. salmonicida* vaccine could be increased by including a preparation (killed *Mycobacterium butyricum* cells) known to enhance cellular immunity. Similar results have also now been found with other substances. Davis and Hayasaka (1984) found that an extract (EtE) from the tunicate *Ecteinascidia turbinata* potentiated the immune response of the American eel (*Anguilla rostrata*) to the pathogen *Aeromonas hydrophila*. Kitao and Yoshida (1986) enhanced the non-specific resistance of rainbow trout (*Oncorhynchus mykiss*) to *A. salmonicida* with a synthetic peptide, administered without the antigen. The efficacy of levamisole as an immunostimulant is also under active investigation (Kajita *et al.*, 1990; Siwicki *et al.*, 1990). Finally, Yano et al. (1989) and Robertsen et al. (1990) reported success at enhancing non-specific immunity against bacterial fish pathogens (*A. hydrophila* and *A. salmonicida*, respectively) with glucans derived from *Schizophyllum commune* and *Saccharomyces cerevisiae*, respectively.

MATERIALS AND METHODS

Experimental Animals

The fish used in the experiments were either coho (*Oncorhynchus kisutch*) or chinook (*O. tshawytscha*) salmon pre-smolts. These fish were reared at the Rosewall Creek Hatchery, Qualicum, B.C., in pathogen-free well water.

Because fish previously exposed to *A. salmonicida* show resistance to rechallenge by the pathogen, it was necessary to confirm that fish used in the studies were naive to this pathogen. Agglutinin levels were assayed to confirm that this was the case. Serum samples having an anti- *A. salmonicida* titre of 4 or less were considered to not have been previously exposed. In no case was there evidence of fish from this facility being exposed to *A. salmonicida* and the facility had a history of freedom from the disease.

Fish were transferred to the Pacific Biological Station in an insulated container in Rosewall Creek Hatchery water at least two weeks prior to the start of experiments. Oxygen was added via air stones during the approximately one hour long transport time to the experimental facility. Upon arrival at the Pacific Biological Station, fish were placed into 650 L flow through aquaria with a dechlorinated, ultraviolet light-sterilized, municipal water supply. The characteristics of this water have been described by Bell *et al.* (1984). The water temperature at the experimental laboratory was adjusted to match the temperature at the Rosewall Creek hatchery. Where an increased water temperature was desired, the temperature was raised by increments of approximately 1 °C per day.

Feed was withheld for a period of two days prior to and two days after transfer of the fish from the hatchery. With the exception of the feed study, the fish used in these experiments were fed a pelleted, commercial dry diet

ad libitum. All fish were maintained on a photoperiod of approximately 8 h light and 16 h dark.

Immobilization of fish during procedures such as fin-clipping, injection and blood collection was achieved using 2 phenoxyethanol (2 PE). The anaesthetic was mixed with aquarium water (1:5 v/v) and shaken vigorously to disperse the compound. This was then added to the water to achieve an approximate final concentration of 1:7000. Salmon exposed to the anaesthetic in this manner showed loss of equilibrium within approximately 30 seconds. The water used for anaesthesia was continuously aerated.

During experiments where it was necessary to maintain different treatment groups within the same tank, groups were identified by fin clipping. Fins used for this purpose were the adipose, the left and right pelvic, and the upper and lower lobe of the caudal fin.

Maintenance of A. *salmonicida* Virulence

Udey and Fryer (1978) have shown that an association exists between the possession of virulence by *A. salmonicida* and the presence of the extra protein layer known as the A-layer in this bacterium. Other workers (Ishiguro et *al*., 1981; Evenberg *et al*., 1988) have demonstrated that certain conditions of culture on laboratory media have resulted in an attenuation of virulence associated with the loss of the A-layer. The use of a virulent pathogen for challenging treatment groups is of utmost importance in assessing the efficacy of a preventative treatment (Amend, 1981). It was therefore necessary to adopt a consistent approach to culturing the organism used for the production of bacterin and for the production of a challenge organism.

Selected and representative fish that had died from an *A. salmonicida* infection were stored at -80 °C until used. When a source of virulent *A. salmonicida* was required, one or two of these fish were thawed at room temperature and kidney material was streaked onto trypticase soy agar (TSA, Difco). The strain of *A. salmonicida* used in all experiments here was strain 76-30 (Olivier *et al.*, 1985a). The plates were cultured at 15 °C for 4 days and a single colony was then used to inoculate either TSA plates or trypticase soya broth (TSB, Difco) to produce sufficient quantities of the bacterium. In the case of the broth culture, a small amount of antifoam B (Sigma) was added. By using the above routine for obtaining virulent *A. salmonicida*, the number of passages on laboratory media was kept to a minimum and ensured consistency in both bacterin and challenge organism production.

With the association between virulence and the A-layer (Adams *et al.*, 1988; Trust *et al.*, 1983), and the necessity of using a virulent strain of *A. salmonicida* as a source of immunogenic bacterin (Olivier *et al.*, 1985b), a means of assuring the presence of A-layer was required. Two simple techniques were used to determine A-layer presence: autoaggregation and the Coomasie dye test.

Autoaggregation

The A-layer causes the autoaggregation of *A. salmonicida* (Udey and Fryer, 1978). This trait was therefore used as an assay to indicate its presence. A dense suspension of *A. salmonicida* in sterile saline was homogenized with a glass mortar and Teflon pestle. The homogenized cell suspension was adjusted to an absorbance of 2.0 at 540 nm on a Perkin Elmer UV/Vis spectrophotometer and allowed to stand for 30 minutes at

room temperature. Suspensions that aggregated and began to settle out of suspension within 30 minutes were considered to be positive for autoaggregation. Those suspensions that remained as a stable suspension were considered to be non-autoaggregating and therefore unsuitable for use in the present study. Similar techniques were used by Sakai and Kimura (1985) to assay for autoaggregation of this bacterium.

Coomasie Dye Test

Cipriano and Bertolini (1988) demonstrated that *A. salmonicida* strains with the A-layer (A^+) and strains without this layer (A^-) could be distinguished from each other by the addition of 0.01 % of the protein specific dye Coomasie Brilliant Blue to the culture medium. Because the A-layer is a protein (Kay *et al.*, 1981), the blue dye is strongly retained by A protein positive colonies. The colony then takes on a dark blue appearance. Compared to colonies of A-layer positive strains of *A. salmonicida,* colonies of the A^- strain are faint blue in appearance.

A. salmonicida cells to be used for challenging fish were suspended in a sterile solution of 0.1% peptone and 0.85% saline (peptone-saline). A drop-plate count (Miles and Misra, 1938) was performed on this suspension using TSA supplemented with 0.01 % Coomassie Brilliant Blue dye. The counts were expressed as cfu·mL^{-1} in the challenge suspension and as % virulent (dark blue) *A. salmonicida* in this suspension. In all cases, the technique described above for maintaining virulence gave 100% dark blue (A^+) colonies.

To verify the purity of the challenge organism as *A. salmonicida*, an aliquot was taken for culture at the time of challenge. This was inoculated

onto TSA and grown at room temperature for 2 to 3 days. The suspension was considered to be *A. salmonicida* if the colonies isolated were morphologically similar to *A. salmonicida*, and a sample of these colonies were Gram-negative, non-motile, coccobacilli, and produced a reddish-brown diffusible pigment. As an additional measure, a slide agglutination test was conducted on cells from a representative colony using rabbit anti-*A. salmonicida* antiserum (Gibco).

Challenge Procedure

Immersion Challenge

An injection challenge method offers the advantage of ensuring that an equal number of infectious organisms has been administered to each fish. However, this method bypasses most of the innate physical and chemical barriers to infection (Ellis, 1981; Ingram, 1980). In addition, this method may not fully or realistically assess the immune status of an individual fish. It is possible that only localized immunity (Georgopoulou and Vernier, 1986; Salmon, 1986; Hart *et al.*, 1988) in the peritoneal cavity is being measured by introducing the challenge organism here. This may be particularly true of vaccination experiments where the antigen is also injected i.p. An immersion challenge method has been proposed by others (McCarthy, 1983; Amend, 1981) as a more realistic approach to efficacy testing of prophylactic treatments.

A. salmonicida was grown in aerated trypticase soy broth (TSB, Gibco) at 15 ^{o}C for 4 days. Dilutions of this broth were made in peptone-saline and the absorbance determined at 540 nm in a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer. A dilution giving an absorbance of between 0.2 and 0.8 was used to back-calculate to that of the original broth suspension. Since

1.0 absorbance units = 5 x 10^8 cells·mL^{-1}, the culture broth could be diluted to give an approximate desired concentration in the challenge suspension. A sample was taken directly from the challenge container to confirm the actual cells·mL^{-1} by the drop-plate method.

The immersion challenge apparatus consisted of a 4 L perforated bucket fitted within a 30 L vessel. All fish could be placed into and removed from the challenge bath by simply removing the perforated inner bucket.

Fish were netted from their holding tank and placed in a clean vessel containing the perforated inner bucket. The inner bucket containing the fish was then removed and placed into the challenge suspension. The fish were exposed to the pathogen in this manner for 15 min. with oxygenation. At the end of the exposure period, the fish were removed by lifting out the inner bucket. The fish were then placed in a tank where they were observed for mortalities.

Due to the presence of antibacterial substances in fish mucus (Cipriano and Heartwell, 1986; Ellis, 1981; Ingram, 1980), a fresh challenge suspension was used for each batch of fish challenged. All buckets were well rinsed with dechlorinated tap water between challenge batches but they were not treated with detergents or disinfectants.

Cohabitation Challenge

A water-borne method of challenge better approximates a natural infection than injection challenge because the pathogen has to penetrate all of the host's impediments to infection. However, the concentration of pathogen required to produce a sufficient challenge upon a single, brief exposure is well above what one might expect to find in the natural

environment. A more natural method of challenge is cohabitation challenge. This method relies on a fish-to-fish transmission of the pathogen without the use of artificial methods or unrealistic bacterial concentrations. Conceptually, at least, such a means of infection better approximates the natural infection process.

Three groups of 30 fish each were injected i.p. with graded doses of *A. salmonicida*. Each of the three groups received 5, 50, or 500 *A. salmonicida* cells per fish, as determined by the absorbance method. These fish (90 in all) were distinguished from experimental groups by a small V-shaped clip to the right operculum and added to the tank of vaccinated fish. A furunculosis "epizootic" was then allowed to proceed. By using three levels of challenge in the "infection source" fish, a prolonged exposure to "natural" levels of pathogen was achieved.

Statistical Analysis

Mortality data were analyzed for statistical significance using chi square contingency table analysis. When appropriate, Fischer's exact test was used. These statistical methods are described in Zar, 1984.

Efficacy Trials With Seven Candidate Immunostimulatory Substances.

The ability of various immunostimulants to potentiate the immune response of coho salmon to *A. salmonicida* bacterin was investigated. Several immunostimulants were identified from the literature (Table 1) based on their reported ability to potentiate the cellular portion of the immune system. Immunity to *A. salmonicida* is believed to be effected largely by these mechanisms (Smith *et al.*, 1980). The purpose of this study, therefore,

was to investigate whether these candidate immunostimulants were capable of enhancing the anti-*A. salmonicida* protection afforded by a simple *A. salmonicida* bacterin.

A. salmonicida bacterin was combined with the agents in Table 1 and emulsified in modified Freund's incomplete adjuvant (MFIA) using a Teflon mortar and pestle as per Olivier *et al.* (1985a). The concentration of bacterin in each of the test vaccines was 10 $mg{\cdot}L^{-1}$ (wet weight) with the appropriate amount of immunostimulant being added to achieve the doses shown in Table 1 when delivered using a 0.1 mL injection. The form of VitaStim-Taito used in this particular study was a highly purified and depolymerized preparation with an approximate molecular weight of 4.75×10^5.

For each treatment, there were two replicates. Each replicate consisted of 25 fish, each of which received a single i.p. injection of the test vaccine. Control groups were injected with the bacterin emulsified in MFIA only. The fish within a given treatment were identified with fin clips unique for that particular treatment. Replicates of the combined treatment groups were held in two separate flow-through 650 L aquaria where they were maintained at 13 ± 1 ^{o}C and fed a commercial dry diet ad libitum throughout the study.

The coho salmon used in this study (mean weight = 10.8 g) were vaccinated on August 11, 1987. A period of time was allowed to elapse so that an immune response could be mounted to the *A. salmonicida* antigen. On September 17, 1987 (27 days post vaccination) the fish were challenged as outlined below. The first replicate was challenged by a 15 min. immersion in 4.6×10^5 $cfu{\cdot}mL^{-1}$ of virulent *A. salmonicida*. The second replicate was challenged by a cohabitation method as described above.

Mortalities were monitored until October 7, 1987 in the immersion challenged group and until October 23, 1987 in the cohabitation challenged group (20 and 36 days post challenge, respectively). The difference in the length of monitoring time between the two types of challenge was a result of the different infection processes. In the group of fish challenged by immersion, the infection was a direct process. However, in those fish receiving a cohabitation challenge, a period of time had to be permitted to allow fish-to fish transmission of infection to occur.

Some 10 percent of the mortalities were selected at random so that cause of death could be verified. Furunculosis was considered to be the cause of death when short, Gram-negative, rod-shaped bacteria with slightly polar staining characteristics were evident in Gram-stained kidney smears, when pure cultures of the pigment-producing bacterium were recovered on TSA from the kidney samples, and when clinical signs in the dead fish were compatible with those caused by *A. salmonicida.*

Immunostimulants:

The immunostimulants used in this study were obtained from several sources. VitaStim-Taito (VST) was donated by Taito Co., Ltd (Tokyo, Japan). The product used in this study was a highly purified and partially depolymerized product with a molecular weight of 4.75×10^5. Lentinan was supplied by Ajinomoto Pharmaceutical Co., Ltd. (Tokyo, Japan). Wy-18,251 was supplied by Wyeth Laboratories, USA. Levamisole and diethyl-dithiocarbamate (DTC) were purchased from Sigma (St. Louis, MO). Modified Freund's complete adjuvant (MFCA) was prepared according to the methods of Olivier et al. (1985a). The *M. butyricum* used in the preparation was a killed and dried product purchased from Difco (Detroit, MI).

Renibacterium salmoninarum cells used to make the *R. salmoninarum* bacterin (RSB) were grown on KDM-2 agar (Evelyn, 1977) at 15 ^{0}C for 20 days. The cells were harvested, suspended in 0.5% formal-saline overnight at room temperature, and washed three times in sterile saline (0.85%). The suspension was kept refrigerated until use (within one week).

Antigen Preparation:

The *A. salmonicida* bacterin, used as the antigen source in this experiment, was produced by exposing a suspension of live, virulent *A. salmonicida* to a solution of 0.3% formalin-saline solution overnight at room temperature. The resulting suspension was washed 3 times with sterile saline. The *A. salmonicida* used was the A-layer positive strain AS76-30 and it had been cultured for 4 d at 15 ^{o}C on TSA

Table 1. Immunostimulants used for the initial screening study. These substances were administered by injection in an admixture with *Aeromonas salmonicida*.

COMPOUND	REFERENCE	DOSE USED
VitaStim-Taito	Komatsu, 1974	15 $mg \cdot kg^{-1}$
Lentinan	Hamuro *et al.*, 1984	5 $mg \cdot kg^{-1}$
Modified Freund's Complete Adjuvant	Olivier *et al.*, 1985a	5 $mg \cdot kg^{-1}$
Diethyldi-thiocarbamate	Renoux and Renoux, 1984	12.5 $mg \cdot kg^{-1}$
R. salmoninarum bacterin	Amend and Johnson, 1984	92.6 $mg \cdot kg^{-1}$
Wy-18,251	Gregory, 1984	10 $mg \cdot kg^{-1}$
Levamisole	Amery and Horig, 1984	5 $mg \cdot kg^{-1}$

ıce of VST, Lentinan, and *Renibacterium salmoninarum* Bacterin on anti-*Aeromonas salmonicida* Agglutinin Response

Coho salmon (mean weight 22.5 ± 1.2 g) were vaccinated with 0.1 mL of an emulsion (MFIA) containing the *A. salmonicida* bacterin and either VitaStim-Taito (VST), Lentinan, or RSB. Control groups received 0.1 mL of the bacterin alone in MFIA or MFIA without bacterin.

The variously treated fish (10 fish per group) were placed in a 135 L flow-through aquarium and held at 13 ± 0.5 °C for 25 days until blood collection. Fin clips were used to denote the various treatments.

Blood Collection

Fish were lightly anesthetized with 2-PE during collection of blood. It was advantageous to use only lightly anesthetized fish as this maintained vigorous cardiac output, thus facilitating the collection of a sufficient volume of blood.

Blood was harvested by severing the caudal peduncle with a sharp scalpel and collecting the blood from the caudal vessel in a 370 μL heparinized capillary tube (Fisher). The collected blood was centrifuged at 11,700 RPM for 3 minutes in an Autocrit II (VWR Scientific) centrifuge. Using a diamond-tipped knife, the capillary tube was scored just above the buffy coat layer and the tube was broken along this score line. The plasma fraction was placed into 1.5 mL polypropylene microcentrifuge tubes, capped, and stored at -20 °C until used for the agglutinin assay.

Antigen Preparation

Antigen (*A. salmonicida* bacterin) used to vaccinate fish in this study was prepared from a 4-day old culture of A-layer positive *A. salmonicida* (strain AS76-30) grown on tryptic soy agar (TSA, Gibco) at 15 ^{O}C. The cells were collected and suspended in 0.85% saline to which formalin was added (0.3% final concentration). This suspension was allowed to stand overnight at room temperature. The killed cell suspension was washed three times in sterile saline prior to injecting it into the test fish.

Due to autoaggregation of A-layer positive *A. salmonicida,* a non-autoaggregating antigen preparation was used for determining the agglutinin titre so that false positive results would be avoided. For this purpose, an A-layer negative variant of strain AS76-30 was prepared by passing the strain several times on TSA and growing the bacterium at room temperature for several passes. The strain so produced, was non-autoaggregating within 30 min. and did not stain blue on CBB agar.

Strain AS76-30 (A^{-}) was grown on TSA at room temperature and harvested in sterile saline. The resulting cell suspension was killed by heating for 30 minutes in a 70 ^{o}C water bath. The killed *A. salmonicida* (A^{-}) cells were washed 3x in sterile saline and resuspended to an absorbance of 2.5 at 420 nm. This suspension was used in the microtitre agglutination test.

Antibody Titre

The antibody titre was determined using standard microtitre agglutination methods. Each well of a standard, 96 well microtitre plate was filled with 25 µL of saline and an additional 25 µL of plasma sample was added to the first well of a series. Plasma samples were run in duplicate.

Using 25 μL microdiluters (Cooke, USA), serial two-fold dilutions were made in each series. The last well received saline in place of plasma and served as a negative control. A four fold dilution of rabbit anti- *A. salmonicida* antiserum (Gibco) was used as a positive control. Positive controls were run once, in duplicate, for approximately every twelve samples. After the serum samples were subjected to the serial dilution procedure, 25 μL of a well-shaken suspension (absorbance = 2.5) of *A. salmonicida* (A^-) was added to all of the wells on the 96 well plate. The microtitre plates were then covered and placed in a 15 °C incubator overnight.

The following day, the plates were read to determine the agglutinin titre. To ensure that differences in lighting did not result in differences in the interpretation of agglutination, the plates were illuminated from beneath with a light source. Wells showing a thin film of cells on the bottom were considered to be positive for agglutination. Those wells in which the cells settled into a tiny "button" at the bottom of the wells were considered negative for agglutination. The appearance of the "button" was defined by the negative controls which were also used to verify that autoagglutination had not occurred. The appearance of wells showing agglutination in the positive controls (rabbit anti-*A. salmonicida* serum) was compared to dilutions of plasma samples scored as positive for agglutination. The agglutinin titre was taken to be the reciprocal of the highest dilution showing agglutination.

Statistical analysis of the agglutination data was performed using Student's t-test with an MS-DOS microcomputer using the Statgraphics statistical analysis program.

The Influence of VitaStim-Taito on the Nonspecific Immune Response of Juvenile Coho Salmon

Komatsu (1974) demonstrated that in mice, the administration of purified VitaStim-Taito (VST) resulted in the activation of macrophages. Furthermore, this activation resulted in an increased level of resistance to challenge by the pathogen *Mycobacterium tuberculosis*. Immunity to this pathogen, like *A. salmonicida*, is considered to be primarily cellular in nature. An experiment was therefore conducted to determine if the apparently nonspecific form of immunity observed by Komatsu (1974) could be applied prophylactically against the fish pathogen *A. salmonicida*.

To determine the immunizing effects of VST in juvenile coho salmon (mean wt. 5.22 $\pm$ 0.13 g) and the duration of any resulting immunity, an experiment was conducted as follows. Two replicates, each containing 30 fish, received the crude preparation of VST (designated VSTm) administered in the form of a 0.1 mL i.p. injection. VSTm was administered at a rate of 20 $mg{\cdot}kg^{-1}$ fish, emulsified in MFIA. This form of VST has an approximate molecular weight of 1-2 x 10^6. Control groups received either an equal volume of *A. salmonicida* bacterin (1.0 $mg{\cdot}fish^{-1}$) emulsified in MFIA or MFIA only.

Fish held at 17 ^oC were vaccinated on May 17, 1988, and challenged 20 days later by a 15 min immersion in 5.3 x 10^5 $cfu{\cdot}mL^{-1}$ of wild-type AS76-30. Mortalities were followed until July 3, 1988. Randomly selected mortalities were determined to have died from furunculosis based on the previously described criteria.

Assessment of the Efficacy of Immersion Administered VitaStim-Taito With and Without Antigen

Fifty chinook salmon (mean weight = 6.49 $\pm$ 0.41g) were immersed in various experimental vaccines for 15 min with oxygen. A 15 min exposure had been reported as adequate for antigen uptake (Tatner, 1987). Two forms of VST were tested. These were the highly purified and partially depolymerized form of VST (molecular weight 4.75×10^5) and the crude form of VST (MW = $1\text{-}2 \times 10^6$) consisting of extracellularly excreted VST and colloidally pulverized mycelia (designated as VSTp and VSTm, respectively). Fish were exposed to either (1) water, (2) antigen only, (3) antigen plus purified VST (100 $mg \cdot L^{-1}$) or (4) antigen plus crude VST (150 $mg \cdot L^{-1}$). The antigen used was a ten-fold dilution of a formalin-killed, *A. salmonicida* broth culture grown for 4 d at 15 ^0C in TSB. Both forms of VST were also administered by themselves as controls. No anaesthetic was used prior to or following the immersion in broth. An additional group of 50 fish was injected i.p. with the formalin-killed *A. salmonicida* broth culture to check on the immunocompetence of the test fish. Feeding of the fish used in this study was withheld for 2 days prior to and 2 days following vaccine exposure.

After exposure, the fish were held in separate, 40 L static (aerated) aquaria maintained at 13 ^oC for two days and then fin clipped, under anaesthesia. Combinations of various fins were clipped according to the treatment received. Fish from each treatment group were separated into two replicates and each replicate was placed in separate 135 L flow-through aquaria and allowed to respond to the immunization treatment for a total of 21 d post-immunization. The fish were challenged by immersion in 2.5×10^5 $cells \cdot mL^{-1}$ of virulent *A. salmonicida* for 15 min. with added O_2. Randomly

selected mortalities were assayed for the presence of *A. salmonicida* in kidney tissue.

Assessment of the Prophylactic Efficacy of VitaStim-Taito when Administered via the Feed

Diet Preparation

Artificial diets were formulated containing four different levels of VitaStim-Taito: 0, 0.01, 0.1, and 1.0 % VST in the salmon diet (designated diets # 1, 2, 3, and 4 respectively). The crudest form of VST available was used in these studies as this form has the lowest production cost and the highest molecular weight. Higher molecular weight forms of VST provide greater biological activity in mice as measured by antitumor activity (Kojima *et al.*, 1986). The preparation used here consisted of a dried, spent, culture broth and colloidally pulverized VST-containing mycelia (VST MW = 1-2 x 10^6). A basal diet was prepared by first mixing the dry ingredients (Table 2) together for 15 minutes to ensure homogeneity. Next, the liquid constituents were added, and mixing was continued for a further 15 minutes. The vitamin and mineral supplements (Tables 3 and 4, respectively) were prepared separately as premixes with α-cellulose as a carrier. Additional premixes were prepared separately containing the various levels of VST, with α-cellulose forming the non-VST volume of the premix. Both α-cellulose and VST are indigestible to salmonids which prevents the possibility that there is a difference in available energy and thus nutritional status between the four diets tested in each of the experimental groups. The premixes were added to the basal diet prepared above and mixing was again continued for 15 min. The diets, so prepared, were identical to one another--differing only in the amount of VST.

Each of the four diets were steam pelleted separately and allowed to cool and dry. Herring oil was then sprayed onto the surface of the pellets (77.08 $g \cdot kg^{-1}$) with an airless spray gun while rotating the pellets in a motorized cement mixer. The feeds were stored tightly wrapped in plastic bags at -20 °C until use.

Experimental Design - Oral Administration of VitaStim-Taito

Groups of chinook salmon (3.71 $\pm$ 0.13 g) were each placed into 30 L flow through tanks (50 $fish \cdot tank^{-1}$) and held at 15.5 $\pm$ 0.5 °C for 3 weeks. During this time, all of the experimental groups were fed the non-VST-containing basal diet to allow them to adjust to the feed and to acclimate to their environment. Prior to challenge, the fish were fed one of the four test diets for 7 days at approximately 2% body weight per day. To prevent error due to the presence of anorexic fish, 10 of the 50 fish were removed from each tank prior to challenge. Fish that appeared emaciated were selected for removal.

The remaining 40 fish per replicate (3 replicates for each treatment) were challenged on day 7 by immersion for 15 min in 6.8×10^4 $cells \cdot mL^{-1}$ of virulent *A. salmonicida*. Throughout the post-challenge period, experimental diets continued to be fed to the respective groups.

In a concurrent experiment, three additional replicates for diets 1 and 4 and two additional replicates for diets 2 and 3 were similarly treated but challenged by immersion for 15 min in 2.4×10^3 $cells \cdot mL^{-1}$ of *Vibrio anguillarum* to assess the ability of VitaStim-Taito to prevent mortality due to other pathogens. The *V. anguillarum* isolate (R-20 strain) was obtained from a chinook salmon epizootic at a commercial saltwater net pen site in British

Columbia and was part of a collection of bacterial fish pathogens held at the Pacific Biological Station, Nanaimo, B.C.

Table 2. Composition of the basal diet used in the oral administration trials. Four experimental diets containing four levels of VST were prepared from this basal diet.

Constituent	$g \cdot kg^{-1}$ diet
Steam Dried Herring Meal	558.66
Dried Whey	84.6
Blood Flour	48.17
Euphasids (whole, frozen)	21.02
Wheat Middlings	126.19
Herring Oil	14.3
Vitamin Supplement	18.414
Mineral Supplement	18.414
Choline Chloride (60% active ingredient)	4.603
Ascorbic Acid	1.84
Permapell$^{(TM)}$	13.81

Table 3. Composition of vitamin supplement premix used for VST feed study. The vitamin premix was prepared separately and then added to the basal diet.

Vitamin	$g \cdot kg^{-1}$ as fed
D-Ca pantothenate	0.178
pyridoxine hydrochloride	0.0414
riboflavin	0.0582
niacin[1]	0.280
folic acid	0.0212
thiamine mononitrate	0.0374
biotin	0.138
B_{12}	0.055
hetrazone	0.1068
vitamin E	1.105
vitamin D_3	0.00442
vitamin A	0.0184
inositol	0.368
α-cellulose	16.001
	18.413

[1]added last

Table 4. Composition of mineral supplement premix used for VST feed study. The mineral premix was prepared separately and then added to the basal diet.

Mineral	$g \cdot kg^{-1}$ as fed
$MnSO_4 \cdot H_2O$	0.212
$ZnSO_4 \cdot 7H_2O$	0.202
$CoCl_2 \cdot 6H_2O$	0.0037
$FeSO_4 \cdot 7H_2O$	0.344
KIO_3	0.0155
NaF	0.0204
Na_2SeO_3	0.00020
NaCl	3.510
$MgSO_4 \cdot 7H_2O$	2.948
α-cellulose	11.139
	18.413

RESULTS

Efficacy Trials With Seven Candidate Immunostimulatory Substances.

Results of the trials dealing with the various immunostimulants are summarized in Table 5. Three of the seven immunostimulants tested (VST, lentinan, RSB) improved survival, irrespective of the challenge method used. In the treatment groups challenged by immersion, a statistically significant improvement in survival over that of the group receiving bacterin alone was noted in fish administered bacterin combined with either VST, lentinan, DTC, RSB, or MFCA. In treatment groups challenged by the cohabitation method, a similar result was observed with the fish vaccinated with VST, lentinan, or RSB in combination with bacterin over that for controls receiving bacterin alone. Those fish receiving MFCA or DTC combined with antigen failed to show a significant improvement in survival over that of the respective control group. Highly variable results were seen with Wy-18,251 with performance equivalent to that of VST or lentinan in one replicate but no better than the control-vaccinated fish in the other replicate.

Table 5. Results of screening of various immunostimulants for their ability to potentiate the immune response to *A. salmonicida* bacterin.

CHALLENGE TYPE	TREATMENT RECEIVED	#DEAD/ #START	% MORTALITY	RP[1]	$p < 0.05$[2]
	MFCA	19/25	76	1.3	Y
	VST	18/28	64	1.5	Y
	LENTINAN	16/25	64	1.5	Y
IMMERSION	LEVAMISOLE	21/25	84	1.1	N
	DTC	17/25	68	1.4	Y
	RSB	9/25	36	2.7	Y
	Wy-18,251	23/25	92	1.0	N
	CONTROL	24/25	96	1.0	-
	MFCA	20/25	80	1.0	N
	VST	13/25	52	1.5	Y
	LENTINAN	13/25	52	1.5	Y
COHABI-	LEVAMISOLE	15/25	60	1.3	N
TATION	DTC	14/25	56	1.4	N
	RSB	11/25	44	1.8	Y
	Wy-18,251	13/25	52	1.5	Y
	CONTROL	20/25	80	1.0	-

[1]RP=relative potency = percent mortality in the control group divided by the percent mortality in the treatment group.

[2]Y=significant at the 0.05 level; N=not significant at the 0.05 level by Student's *t*-test.

Effect of Selected Immunostimulants on the Agglutinin Response to A. *salmonicida* Antigen

All fish vaccinated with the antigen, either alone or in combination with an immunostimulant produced an elevated level of agglutinins over that of the controls. Fish treated with modified Freund's incomplete adjuvant without antigen (the controls), developed a titre ranging from nondetectable to 4. However, the groups of fish receiving an emulsion of immunostimulant with antigen or receiving antigen only showed significantly ($P < 0.05$) higher agglutinin titres than the MFIA controls.

The agglutinin titres are summarized in Table 6 Coho salmon vaccinated with *A. salmonicida* bacterin without immunostimulant developed an average agglutinin titre of 50.7. Those fish vaccinated with lentinan or VST in combination with the bacterin developed mean titres ranging from 29.3 for lentinan to 79.3 for the crudest form of VitaStim-Taito, VSTm. The greatest increase in agglutinin titre was observed when RSB was used with the bacterin (mean titre of 136.0). However, because of substantial variations in the degree of agglutinin production between individual fish within a given treatment group, none of these differences was statistically significant.

Repeated titrations of the reference rabbit anti-*A. salmonicida* serum consistently produced a titre of 64. The differences in agglutination titre measured between these individual fish is thus real and not a result of inconsistencies in the agglutination test procedure or subjectivity in reading of wells.

Table 6. Influence of the immunostimulants VitaStim-Taito (VST), lentinan, and *R. salmoninarum* bacterin (RSB) on the agglutinin response to an *A. salmonicida* bacterin (Ag)[1].

TREATMENT APPLIED	MEAN TITRE[2]	SAMPLE SIZE	COMPARE TO ANTIGEN ONLY $p < 0.05$[3]	COMPARE TO SALINE $p < 0.05$[3]
VST + Ag	79.3 (12.8)	11	N	Y
Lentinan + Ag	29.3 (7.4)	9	N	Y
RSB+ Ag	136.0 (47.4)	10	N	Y
Ag Only	50.7 (15.5)	9	-	Y
Saline Control	3.0 (0.5)	9	Y	-

[1] Coho salmon (average weight 22.5 g) were vaccinated intraperitoneally with the indicated preparations and held for 25 days at $13 \pm 0.5\ ^{0}C$ until sampled.

[2] bracketed values represent the standard error of the mean.

[3] Y=significant at the 0.05 level; N=not significant at the 0.05 level by Student's *t*-test.

Stimulation of Nonspecific Immunity by VitaStim-Taito

Chi-square heterogeneity tests were performed on mortality data from all replicate treatment groups. These tests indicated that it was statistically valid to pool all replicates within a given treatment.

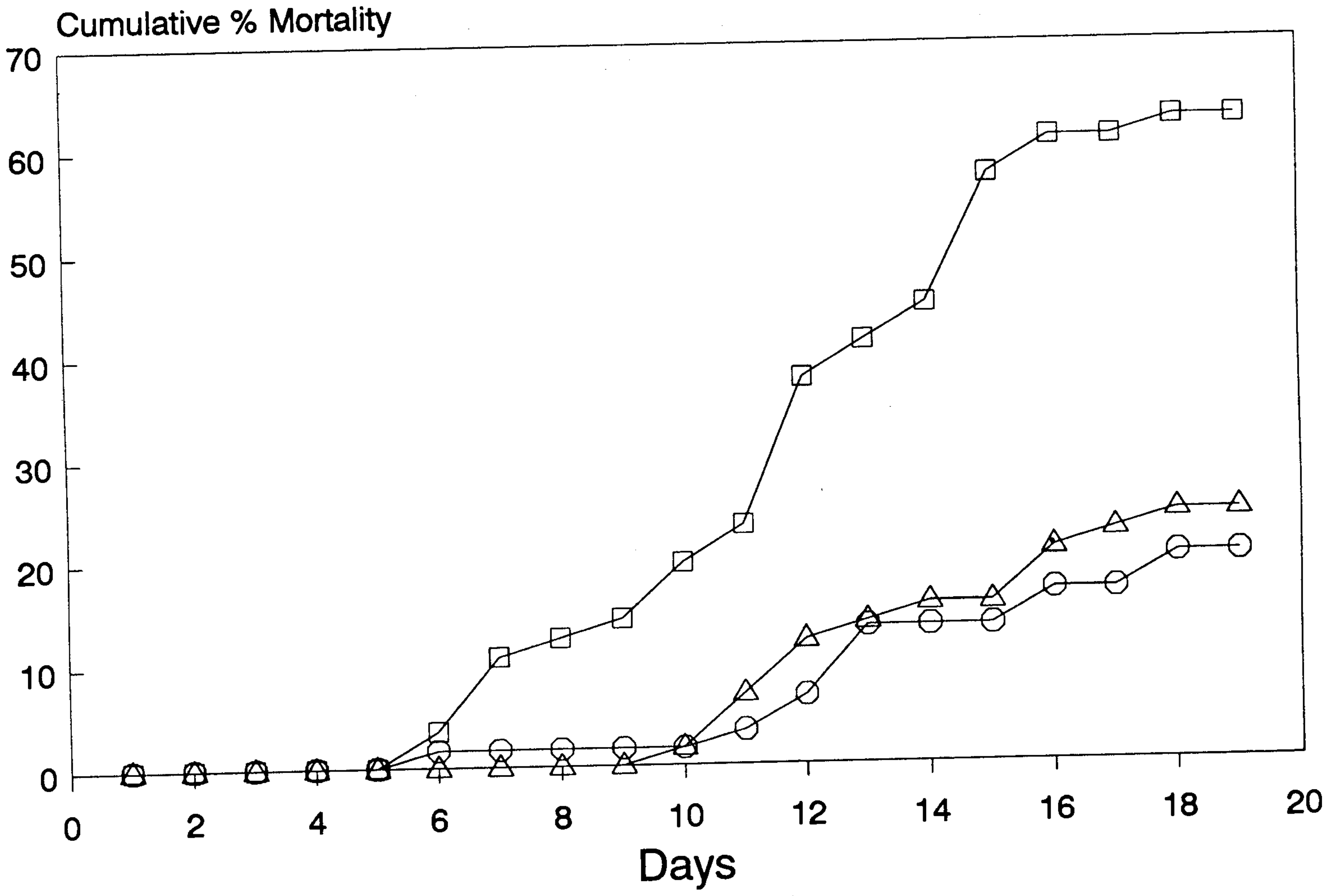
A substantial and significant reduction in mortality (Fig 1) was observed when fish were administered a single i.p. injection of VSTm compared to fish receiving only the carrier. Fish receiving the carrier control experienced a 76.8 % mortality. By contrast, the group of fish receiving the VSTm treatment underwent a much lower mortality of 46.6%. As a comparison, a group of replicate fish vaccinated with *A. salmonicida* antigen was included in this study. This group had the lowest mortality. By the end of the study, the cumulative mortality in the bacterin-injected group had risen to only 26.7%.

It is interesting to note that although at the end of the study the bacterin-inoculated group showed a statistically significant decrease in mortality, the VSTm treated group showed a similar degree of protection up to approximately June 22 (36 d after receiving the single VSTm treatment). The two groups did not differ significantly up to this time. After June 22, the mortality in the VSTm treated group increased somewhat (see Fig 1).

A small number of individuals had died prior to challenge. A Gram stain and culture of the kidney tissue on TSA failed to indicate bacterial infection as a cause of death. Presumably, handling received during injection was the reason for death as all mortalities resulted within a few days of injection. Because these deaths were not believed to be related to the treatment received, they were excluded from the results and from the determination of percent mortality. The exclusion of mortalities not attributable to infection is supported by Amend (1981) and Finney (1971).

Dealing with uninfected mortalities in this manner provided a more accurate picture of the protective effects of treatments relative to the challenge organism.

Fig. 1. Mortalities in coho salmon (*Oncorhynchus kisutch*) treated by injection with saline, VST, or *A. salmonicida* bacterin and challenged 20 days later (day 0) with *A. salmonicida*. Coho salmon (60, 5.22 $\pm$ 0.13 g fish per group) were injected intraperitoneally with 0.1 mL of modified Freund's incomplete adjuvant containing saline (□), crude VST (20 mg·kg-1 fish) (△), or *A. salmonicida* bacterin (200 $mg \cdot kg^{-1}$ fish) (○). Fish were held in fresh water at 17^0C and challenge was by a 15 min immersion in viable *A. salmonicida* cells ($5.3 \times 10^5 \cdot mL^{-1}$).


Cumulative % Mortality
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Days

Immersion Administration of VitaStim-Taito

A summary of the mortalities in each of the treatment groups is shown in Table 7. Relative potencies (Amend, 1981) were calculated because of a difference in the severity of challenge between replicates. The group receiving the i.p. administered bacterin was protected against immersion challenge with virulent *A. salmonicida* to a significantly ($p < 0.05$) higher degree than the control group. Moreover, the level of protection by bacterin was similar between both replicates (relative potency = 3.0 and 3.2 for replicate one and two respectively).

There was no significant change in mortality over that of the controls in any of the experimental vaccines tested. *A. salmonicida* cellular antigen failed to be protective when administered by immersion either alone or in combination with the crude or purified VST. Likewise, no significant difference was observed in mortality between fish immersed in water and fish immersed in either the purified or crude VST without an antigen source.

A small number of fish in some of the groups died during the 2 day post-exposure period. No bacterial pathogens could be isolated from these fish. Because mortalities occurred even in the groups exposed to water only, it is felt that the deaths were due to the handling protocols used rather than to the challenge administered. These mortalities were excluded from the total number of fish at the experiment's start for the purposes of determining the proportions of fish dying and surviving (Finney, 1971; Amend 1981).

Table 7. Influence of VitaStim-Taito in crude (VSTm) and pure form (VSTp) administered by immersion with and without *Aeromonas salmonicida* bacterin (Ag) on survival following immersion challenge with *A. salmonicida.*

REPLICATE	TREATMENT	# DEAD/ # START	% CUMULATIVE MORTALITY	RP[1]
1	Control	16/23	70%	1.0
	VSTp only	16/21	76%	0.9
	VSTm only	18/24	75%	0.9
	Ag only	14/21	67%	1.0
	VSTm + Ag	17/24	71%	1.0
	VSTp + Ag	14/24	58%	1.2
	Ag (i.p)[2]	5/23	22%	3.2
2	Control	9/23	35%	1.0
	VSTp only	10/22	45%	0.9
	VSTm only	7/25	28%	1.4
	Ag only	11/24	46%	0.9
	VSTm + Ag	6/24	25%	1.6
	VSTp + Ag	10/25	40%	1.0
	Ag (i.p)[2]	3/24	13%	3.0

[1]RP=Relative Potency=%mortality (control)/%mortality (treatment) (Amend, 1981).

[2]*Aeromonas salmonicida* bacterin was administered by intraperitoneal injection as a control.

Oral Administration of VitaStim-Taito

The results of the heterogeneity test indicated that it was legitimate to pool the results from each of the three replicates of fish fed the experimental diets and challenged with *A. salmonicida* (Table 8). The mortality results from this experiment are summarized in Tables 10 and 11.

The addition of VitaStim-Taito into the feed at a concentration of 0.1 % or 1.0 % (diets 3 and 4, respectively) proved to be of prophylactic benefit against *A. salmonicida*. The cumulative mortalities for the groups given these diets were only 1.7 % and 5.8 %, respectively. The difference between diets 3 and 4 was not statistically significant. However, the groups receiving the same diet, either without VitaStim-Taito or with VitaStim-Taito at only 0.01 % showed elevated mortality (16.7 % and 27.5 %, respectively) but this difference was not statistically significant. The groups of fish receiving either diets 3 or 4 had significantly lower mortality than those not receiving any VST or those receiving only 0.01% VST in the diet.

Mortalities in the *V. anguillarum* challenges (Table 11 ranged from between 12.5% to 55% in the control replicates. In diet 4, with the highest concentration of VST, one tank showed only a single mortality. However, the two other replicates had mortalities comparable to those of the control groups. No significant difference was observed between the mortalities of fish fed VST mixed with basal diet at any of the concentrations tested and fish fed the basal diet alone. It should, however, be noted that heterogeneity testing indicated that the pooling of results was not statistically permissible (Table 9).

Table 8. Heterogeneity testing of replicate mortality data obtained in the oral administration of VitaStim-Taito (VST) trials (*Aeromonas salmonicida* challenge).

DIET #	COMPARISON[1]	$P < 0.05$ [2]	POOL [3]
1	R1 vs. R2	N	Y
1	R2 vs. R3	N	Y
2	R1 vs. R2	N	Y
2	R1 vs. R3	N	Y
2	R2 vs. R3	N	Y
3	R1 vs. R2	N	Y
3	R2 vs. R3	N	Y
4	R1 vs. R2	N	Y
4	R1 vs. R3	N	Y
4	R2 vs. R3	N	Y

[1]Comparison between the replicates (R1,R2,R3) within a given diet.

[2]Y=significantly at the 0.05 level; N=not significantly different at the 0.05 level.

[3]Y=Legitimate to pool the replicates; N=not legitimate to pool results of the replicates

Table 9. Heterogeneity testing of replicate mortality data obtained in the oral administration of VitaStim-Taito (VST) trials (*Vibrio anguillarum* challenge).

DIET #	COMPARISON[1]	$P < 0.05$ [2]	POOL [3]
1	R1 vs. R2	Y	N
1	R2 vs. R3	N	Y
1	R1 vs. R3	Y	N
2	R1 vs. R2	Y	N
3	R1 vs. R2	Y	N
4	R1 vs. R2	Y	N
4	R1 vs. R3	N	Y
4	R2 vs. R3	Y	N

[1]Comparison between the replicates (R1,R2,R3) within a given diet.

[2]Y=significantly at the 0.05 level; N=not significantly different at the 0.05 level.

[3]Y=Legitimate to pool the replicates; N=not legitimate to pool results of the replicates

Table 10. Survival of juvenile chinook salmon (*Oncorhynchus tshawytscha*) fed diets containing various amounts of VitaStim-Taito (VST) and immersion-challenged with .

DIET #	VST CONCENTRATION	REPLICATE No.	#DEAD/ #START	POOLED REPLICATES[1]	% MORTALITY
		1	10/40		
1	0 %	2	5/40	20/120	**16.7%**
		3	5/40		
		1	15/40		
2	0.01 %	2	10/40	33/120	**27.5%**
		3	8/40		
		1	2/40		
3	0.1 %	2	0/40	2/120	**1.7%**
		3	0/40		
		1	2/40		
4	1.0 %	2	4/40	7/120	**5.8%**
		3	1/40		

[1]Replicates were pooled after performing heterogeneity test.

<u>**Table 11.**</u> Survival of juvenile chinook salmon (*Oncorhynchus tshawytscha*) fed diets containing various amounts of VitaStim-Taito (VST) and immersion-challenged with *Vibrio anguillarum*.

DIET #	VST CONCENTRATION	REPLICATE No.	#DEAD/ #START	POOLED REPLICATES[a]	% MORTALITY
		1	5/40		
1	0 %	2	22/40	43/120	**35.8%**
		3	16/40		
		1	27/40		
2[b]	0.01 %	2	9/40	36/80	**45%**
		1	13/40		
3[b]	0.1 %	2	23/40	36/80	**45%**
		1	19/40		
4	1.0 %	2	1/40	33/120	**27.5%**
		3	13/40		

[a]Replicates were pooled after performing heterogeneity test.
[b]Only two replicates were used in this treatment group due to a shortage of aquaria.

DISCUSSION

The screening trials identified three compounds with apparent immunostimulatory activity, as measured in terms of survival following challenge: VitaStim-Taito, lentinan, and killed, intact *R. salmoninarum* cells. When these immunostimulants were added to *A. salmonicida* bacterin, they resulted in a significantly increased level of protection against the furunculosis agent over that observed with bacterin alone. This effect occurred with each of the two challenge methods used. Three of the remaining four compounds (MFCA, DTC, Wy-18,251) provided results that were inconsistent and one (levamisole) failed to show promise with either of the challenge methods used.

Levamisole has been demonstrated to be effective in elevating immunity in several mammalian studies (Amery and Horig, 1984; Hennessy et al., 1987; Siwicki, 1987). The drug clearly shows promise for use as an immunostimulant in fish on the basis of *in vitro* studies (Siwicki, 1987; Anderson *et al.*, 1989; Siwicki *et al.*, 1989 and 1990), and in two *in vivo* studies in salmonids, it enhanced resistance against two bacterial fish pathogens (Olivier *et al.*, 1985a; Kajita *et al.*, 1990). The lack of efficacy of levamisole in the present study is not entirely in disagreement with the above work. The *in vitro* assays of immunity have yet to be demonstrated to have clinical relevance in fish. Furthermore, in the *in vivo* study of Olivier *et al.* (1985a), levamisole yielded the least striking increase in *A. salmonicida* resistance among three immunostimulants tested, suggesting that levamisole may not be a highly potent immunostimulant. The immune response to levamisole can also be suppressive or stimulatory, depending on the dose and the manner in which it is administered (Anderson et al. 1989). Such

apparent complications in the use of levamisole indicate that more work is needed to determine the appropriate conditions for its use in fish.

Amery and Horig (1984) summarize information concerning the mechanisms believed to be involved in the immunotropic properties of levamisole. Levamisole is known to restore to normal the T-lymphocyte and phagocyte response of an immunosuppressed host. However, this drug apparently has little effect on elevating the level of immune activity in hosts with an adequate immune response. The present experimental design did not involve immunosuppressive treatment. Given that considerable effort was expended to ensure that stress in the experimental stock was avoided, it is not unreasonable to assume that the group of experimental animals used in these investigations had a "normal" immunological status. The immunocompetence of these fish was evidenced by their positive antibody response. It would be worthwhile in this regard to re-examine the efficacy of levamisole at enhancing the immunity of acutely and chronically stressed fish. Stress is a known immunosuppressant (Anderson, 1990) and is often a factor leading to disease in commercially-cultured fish species.

DTC showed low, but statistically significant protection in the immersion challenged groups. In the cohabitation-challenged fish, DTC did show feeble protection but the level of protection was marginal ($p=0.06$). Conceivably, DTC could show some promise with further study but earlier problems with toxicity (unpublished observations, L. Nikl) of DTC were encountered. Mortalities in groups of fish receiving DTC occurred in initial dose range-finding studies. Given that the therapeutic index of DTC is probably high, this compound was judged to be not entirely safe and was likely to encounter difficulties in the drug registration process.

The contradictory results observed with Wy-18,251 in combination with antigen (see Table 5) are difficult to reconcile. The positive protection observed in the cohabitation challenge group was as potent as that afforded by lentinan or VST combined with antigen. On this basis, it may not be entirely appropriate to overlook the potential usefulness of Wy-18,251. Like levamisole, however, Wy-18,251 can be both immunosuppressive and immunostimulatory and it appears to work best on immunocompromised hosts (Gregory, 1984). Such difficulties in its use complicate its application and indicate that considerable further work may be required to address its possible usefulness in fish culture.

The inconsistent results obtained with MFCA also suggest that further studies with MFCA could be abandoned in favour of studies with the three more promising compounds listed above. The successful results obtained with MFCA by Olivier et al. (1985a) are difficult to reconcile with the findings of the present study and it is only possible to speculate on the reason(s) for this. It may well be that stimulation of the macrophages by MFCA (Olivier et al., 1986) tends to be localized and that the challenge route must be the same as the vaccination route if maximum efficacy of the immunostimulant is to be obtained. In the present study the route of administration of the immunostimulant differed from that of the challenge route.

In the present experiment, enhanced protection was consistently obtained in groups receiving intact, washed, formalin-killed *R. salmoninarum* cells in combination with *A. salmonicida* bacterin. This finding is consistent with observations made earlier by Amend and Johnson (1984). On the other hand, Turaga et al. (1987), using an *in vitro* assay (based on plaque forming cell counts) found that *R. salmoninarum* possessed immunosuppressive activity. Their findings, however, were obtained with the "soluble proteins"

produced by intact *R. salmoninarum* cells. The results of the present study with RSB are promising and the properties of the *R. salmoninarum* cells clearly warrant further investigation.

The consistent protection conferred by both VST and lentinan in our trials was not surprising because these agents are chemically closely related to each other (i.e., they are β-1,3 glucans with β-1,6 glycosidic side chains), they are known to possess antitumor activity (Aoki 1984; Sugawara *et al.*, 1984), and they had been reported to produce resistance in carp (*Cyprinus carpio*) against *Edwardsiella tarda*, a bacterial fish pathogen (Yano *et. al.*, 1989). In addition, a similar compound derived from baker's yeast (*Saccharomyces cerevisiae*) has recently been shown to yield protection in Atlantic salmon (*Salmo salar*) against two bacterial fish diseases: coldwater vibriosis and enteric redmouth disease (Robertsen *et al.*, 1990). Evidence suggests that these compounds act to enhance disease resistance by stimulating the macrophage component of the immune system (Yano *et al.*, 1989; Sugawara *et al.*, 1984); a conclusion supported by earlier work conducted with another type of immunostimulant (Olivier *et al.*, 1985a; 1986). However, as discussed below, the mechanism of action of the immunostimulants may be more complex when they function in the presence of a bacterin. When used alone, VST can induce protection equivalent to that obtained with bacterin alone (see Fig. 1 for results with VST for example) but the level of protection produced by combining VST with bacterin is clearly enhanced (Table 5).

The increased protection against *A. salmonicida* challenge seen in the groups treated with bacterin plus VST, lentinan, or RSB occurred despite the fact that no immunostimulant-mediated increases in the *A. salmonicida* agglutinins had occurred (Table 6). Apparently, induced anti-*A. salmonicida*

agglutinins were not responsible for the observed increase in protection that occurred in these treatment groups. These data suggest that the immunostimulants did not enhance resistance to the pathogen by stimulating the B-cell (humoral antibody producing) component of the specific immune system. Instead, the enhanced protection may have been due to the stimulation of another component of this system (the T-cell component) which then interacted cooperatively with the reticuloendothelial system (the macrophages) to bring about the elimination of the pathogen. The cooperative involvement of T-cells and macrophages in the process would not be surprising as VST, for example, is reported to be a T-cell activator in mammals (Sugawara *et al.*, 1984). In addition, *A. salmonicida*-vaccinated rainbow trout produce soluble factors, reminiscent of the interleukins produced by T-cells, which affect the activity of leucocytes and presumably of macrophages (Smith *et al.*, 1980).

Recently, Michel *et al.*, (1990) reported an interesting experiment in which enhanced resistance to furunculosis was obtained in rainbow trout in the absence of *A. salmonicida*-specific antibodies. The protection, thought to be due to the increased levels of polyspecific antibodies, was induced by repeated injections of phosphate-buffered saline. It was postulated that these antibodies exerted their protective effect by serving as opsonins which permitted increased phagocytosis of the pathogen by macrophages. It is not felt that the immunostimulants used in this study functioned by inducing polyspecific antibodies because, based on their failure to enhance *A. salmonicida* agglutinin levels, they did not appear to function by stimulating antibody synthesis.

Having identified some effective, injection-administerable immunostimulants, the study then focused on more convenient and practical

methods such as immersion or feeding. In connection with the foregoing, VST appears to be a particularly promising compound. Lentinan is prepared from the fruiting body of the Shiitake mushroom (*Lentinus edodes*), a popular delicacy, and cannot be grown in fermentation culture. However, VST is derived from the mycelia of another fungus (*Schizophyllum commune*) that is readily produced by fermentation culture. Furthermore, unlike *R. salmoninarum*, which is grown with difficulty and may therefore prove expensive to mass produce, VST can be mass-produced at favorable cost by batch fermentation culture.

A single exposure to VST by bath failed to result in significant protection whether it was administered in an admixture with antigen or on its own. It is thought that uptake of immersion-administered vaccines is an active process occurring primarily across the gill epithelium (Zapata *et al.*, 1987). Apparently, either uptake of sufficient material did not occur during the exposure time used in this study or VST does not lend itself to uptake from water. The positive and consistent protection seen in the i.p.-vaccinated groups demonstrated that the fish were capable of responding to the antigen and that the handling received in conducting the experiment did not block the immune response through a stress mediated effect.

The poor results obtained with bath- administered VST were somewhat surprising. The duration of exposure (15 min) may have been insufficient for VST uptake. VST is a large molecule with molecular weights of 4.75×10^5 and $1\text{-}2 \times 10^6$ for the two forms used. It would be interesting to further assess VST's efficacy at enhancing immersion-administered vaccines in studies employing longer immersion times.

Encouraging results were obtained with VST when administered as a food additive. Incorporation of VST at a concentration of only 0.1 % resulted

in a significant and reproducible increase in survival from challenge with *A. salmonicida*. Less encouraging results, however, were seen with the R-20 strain of *Vibrio anguillarum*. Immunity to *V. anguillarum* is probably more closely related to humoral factors than to cellular ones. In addition, because of the lack of replication in the results with *Vibrio anguillarum* further testing should be done before final decisions as to whether VST is effective against *V. anguillarum* are made. A similar compound was effective against *Vibrio salmonicida* and *Yersinia ruckeri* (Robertsen *et al.*, 1990).

VST's efficacy when administered by injection is not surprising. It is known to possess antitumor activity in mammals (Sugawara *et al.*, 1984) and to confer disease resistance against *Edwardsiella tarda* in carp (Yano *et al.*, 1989) and *A. salmonicida* in juvenile coho (*Oncorhynchus kisutch*) salmon (Nikl *et al.*, 1991). Of more surprise, however, was its effectiveness when administered via a processed and pelletized feed. Some drugs may lose their biological activity when prepared through the steam pelleting process due to thermal instability and the temperatures associated with this process (approximately 90-95 ^{o}C). The biological activity of VST is dependant on the retention of its triple helix conformation (Yanaki *et al.*, 1983). This compound can retain this configuration at temperatures of up to 135 ^{0}C (Yanaki *et al.*, 1985). Such thermal stability makes this compound ideally suited for incorporation into pelletized, mass-produced, commercial fish diets. Other related β-1,3 glucans such as lentinan or M-glucan are known to possess immunostimulatory activity in fish (Yano, *et al.*, 1989; Robertsen *et al.*, 1990; Nikl *et al.*, 1991) but these compounds are less well chemically characterized with respect to their thermal stability.

One of the effector mechanisms apparently influenced by VST is the macrophage. This component of the immune system is thought to play an

important role in defense against *A. salmonicida* (Smith *et al.*, 1980; Olivier *et al.*, 1986). It appears from the foregoing, that for diseases in which the primary mechanisms of immunity are of an antibody nature, the outcome may not be greatly influenced by VST. VST does not appreciably influence the agglutinin response of fish (Nikl *et al.*, 1991). Another serious pathogen of salmonid fishes, *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease is considered by some individuals to be an agent for which immunological defence is primarily effected by the cellular portion of the immune system. This pathogen is considered to be an intracellular pathogen, residing within the macrophage. Destruction of intracellular pathogens is generally associated with macrophage activation (Tizard, 1984). Activation of the macrophage--a known *in vivo* effect of VST (Sugawara *et al.*, 1984; Yano *et al.*, 1989)--may therefore provide protection against pathogens such as *R. salmoninarum*.

Neither VST nor other β-1,3 glucans can be considered to be panaceas for the treatment of certain salmonid diseases. However, on the basis of VST's apparent actions, it is felt that a wide range of fish disease problems could greatly benefit from its use. It is shown here that VST is successful, at least for furunculosis, at providing consistent protection from challenge and that this can be achieved using an inexpensive, crude product administered by a highly practical and economical method.

SUMMARY

The present study evaluated seven substances known from the literature to have immunological activity. VST, lentinan and RSB showed promise at potentiating the protective effects of a simple formalin-killed *A. salmonicida* bacterin-type antigen. Additionally, DTC and Wy-18,251 showed some possibility for further study but due to problems such as toxicity, less than satisfactory efficacy, or inconsistencies in performance, these compounds show less promise for practical use. For reasons of cost and efficacy, VST was clearly the most promising substance tested. On this basis, it was selected for additional studies to determine if more practical delivery methods could be employed.

Application of the *A. salmonicida* bacterin by the immersion method was not effective whether it was administered with or without VST in either of the forms tested. This too was true of VST on its own. While VST's performance by this method may not be surprising, it would be worth testing VST using longer immersion periods, or along with an antigen known to be effective by immersion administration.

The protective antigen of *A. salmonicida*, believed to be proteinaceous, would not likely survive passage through the digestive system of salmon. Therefore, in the absence of antigen-protection technologies such as encapsulation or antacids, it is desirable to use a biological which provides prophylactic benefit by its own virtue. VST was an effective prophylactic agent when administered by the oral route and even when it was processed through the harsh conditions of a commercial-type steam pelleting process. This finding is considered to be preliminary and further work is required to determine the optimal dose, treatment regime to be used, as well as an assessment of the range of diseases for which it may be effective.

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