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Title: INFECTION AND THE IMMUNE RESPONSE INDUCED BY VIBRIO ANGUILLARUM IN JUVENILE COHO SALMON (ONCORHYNCHUS KISUTCH)

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Immunity in juvenile coho salmon (Oncorhynchus kisutch) was evaluated after immunization against vibriosis by parenteral, oral or immersion methods. Relative levels of immunity were determined by protection of fish from experimental water-borne challenges with Vibrio anguillarum and by serum agglutination titers. Coho salmon eggs were exposed to bacterin during artificial spawning or as embryos 4-5 days before hatching. Two of the six injected embryos had agglutination titers 218 days later, and as juveniles these two fish demonstrated an anamnestic response following intraperitoneal injection of bacterin. Fish that had selected portions of their body immersed in bacterin had high levels of protection and lower agglutination titers when compared to those completely submerged in the same preparation. Results showed that parenterally immunized fish had high titers and a high level of protection, those immersed in the

bacterin had lower titers but a high level of protection and those orally immunized had no serum agglutinins and only moderate protection.

Previously unimmunized fish were significantly protected when passively immunized with coho salmon anti-*V. anguillarum* immunoglobulin partially purified by gel filtration. The agglutination titer of the partially purified antibody was 1:4. Serum or spleen cells obtained from orally immunized fish conferred no protection to fish injected with these substances. Serum from parenterally immunized fish, however, conferred a high level of protection in passively immunized fish and spleen cells from these same donors also provided moderate protection to previously nonimmune recipients. Coho salmon radiolabeled tetrameric immunoglobulin purified by ion exchange and gel filtration was effectively absorbed in the blood after it was intraperitoneally injected. The kinetics of absorption were related to water temperature with less immunoglobulin absorbed at the lower temperatures.

Effects of temperature on infection were examined at seven water temperatures in 3°C increments from 3 to 21°C. The mean day to death and total mortality were related to temperature and a shorter mean day to death and higher mortality was observed at increased temperatures. The effect of temperature on the time of onset of agglutinating antibody formation in parenterally immunized fish was studied in fish held at 6, 12 and 18°C. Titers were first observed on days 10, 15 and 25 in fish held at 18, 12 and 6°C, respectively. Growth curves

of V. anguillarum cultured in Brain Heart Infusion broth were determined at the same three temperatures with growth rates directly related to temperature.

Infection and the Immune Response Induced by *Vibrio anguillarum* in Juvenile Coho Salmon (*Oncorhynchus kisutch*)

by

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INTRODUCTION

Host-pathogen interactions in vertebrate animals can take several courses. Frequently, the process leads to an infection of the host by the pathogen resulting in proliferation of the pathogen within host tissues. The initial infectious event and subsequent host-pathogen processes are dependent upon many host, pathogen and environmental factors. The outcome of these processes may lead to death of the host or recovery with various levels of immunity to a recurrent infection. Immunization is a method to induce an immune response and thus afford protection in an animal before its first encounter with a pathogen.

Mass immunization as a technique for control of infectious diseases in cultured fish depends on the inducement of protection in a large number of animals under conditions that often favor the pathogen. The technology for immunization of intensively propagated fish has emphasized methods that minimize handling and stress to the animal, while at the same time inducing immunological responses that afford high levels of protection. Coho salmon (Oncorhynchus kisutch) are a natural host for the bacterial fish pathogen, Vibrio anguillarum, and together they comprise a host-pathogen system that lends itself to laboratory studies and that is relevant to fish cultural activities. The first commercially available bacterin for salmonid fish was a

product for immunizing these fish against vibriosis, and since that product became available, others have appeared on the market. Various immunization delivery systems have evolved along with these bacterins.

The intent of several of the studies reported here was to evaluate three diverse methods, parenteral, oral and immersion, to deliver antigenic material to fish. Regarding this evaluation, there were three goals. One goal was to compare the efficacy of protection, the ultimate measure of immunity, induced by the different methods. A second goal was to describe the nature of immune responses elicited by these treatments and to quantitate humoral antibody production in response to these immunization techniques. Finally, continued efforts were made to characterize the machinery of the immune system of the coho salmon with particular attention given to the detection of mucus immunoglobulin.

While immunity can profoundly affect the outcome of an interaction between a pathogen and host, environmental temperature also has a powerful influence on infectious processes in ectothermic vertebrates. Pacific salmon favor water temperatures in a range where many host responses and proliferative capacities of pathogens are in a delicate balance. Extremes of temperature can tip this balance in favor of one or the other. A description of the effects of temperature on specific parameters of infection and the immune response were examined in the second part of the study. The in vitro growth dynamics of V. anguillarum and its ability to induce mortality in juvenile coho salmon

at selected temperatures were two parameters of infection studied. The appearance of V. anguillarum specific agglutinins and the kinetics of absorption of tetrameric immunoglobulin from the peritoneal cavity at selected water temperatures were examined as immunity related studies.

LITERATURE REVIEW

Vibriosis of Fish

Observations of vibriosis-like hemorrhagic conditions in eels dates back to the 15th century. Further descriptions were made of a "Red Pest" disease of eels held in sea-water ponds along the European coastline. Early isolations and descriptions of the causative agent were made by Canestrini (1893) and Bergman (1909). A similar bacterium was described in several species of marine and migratory fish and the implicated agents were given various names. A review (Anderson and Conroy, 1970) of the history and geographical distribution of affected species showed that vibriosis was worldwide in numerous species of anadromous and marine fish. Hendrie, Hodgkiss and Shewan (1971) proposed that many of these isolates be combined as a single species, Vibrio anguillarum.

The eighth edition of Bergey's Manual of Determinative Bacteriology (1977) includes a description of Vibrio anguillarum. The organism is a Gram-negative, curved or straight rod motile by a single polar flagellum. It is oxidase-positive, facultatively anaerobic and ferments glucose without gas production. The bacterium has a sodium chloride requirement and is sensitive to 2,4-diamino-6,7-diisopropyl pteridine (O/129) and novobiocin.

Serological relationships of three V. anguillarum isolates from herring and salmonids in the Pacific Northwest were compared to other marine vibrios pathogenic for fish (Pacha and Kiehn, 1969).

Three serotypes were described and the organism isolated from salmonids in the Pacific Northwest was designated serotype 1. In 1973 (Harrell et al., 1976) a second biochemical and serological type was isolated from pen-reared coho salmon (Oncorhynchus kisutch) in Puget Sound, Washington. The fish were intraperitoneally vaccinated with a heat-killed bacterin prepared from the isolate previously implicated in vibriosis epizootics in that region (Harrell, 1978). Ransom (1978) characterized two Vibrio spp. isolated from salmonids held in Yaquina Bay, Oregon that were similar to the two Puget Sound organisms. They were designated Vibrio anguillarum, serotypes I and II. Strout, Sawyer and Coutermarsh (1978) have described a third V. anguillarum serotype from fish on the Maine-New Hampshire coast that was pathogenic for coho salmon.

External pathological signs early in the disease of salmonids include anorexia, discoloration of the skin and inactivity. Late in infection erythema is often seen at the base of the fins, around the vent, within the mouth and on the body surface (Fryer, Nelson and Garrison, 1972). Hemorrhaging occurs at the gills and internally in the viscera (Cisar and Fryer, 1969), and the gut is usually inflammed. The blood picture in experimentally infected coho salmon is characteristic of a hemorrhagic septicemia (Harbell, Hodgins and Schiewe, 1979). Pathogenesis studies in experimentally infected salmonids (Ransom, 1978) indicated that the organism entered the fish by penetrating the mucosa of the lower intestine and rectum.

Target organs after infection were the blood, loose connective tissue, kidney, spleen, posterior gastrointestinal tract and gills.

With efforts to exploit marine and estuarine environments for fish cultural activities during recent decades, came the realization that vibriosis could be the determining factor for the success or failure of such endeavors. In Japan the disease has been important to fish culture in both marine and freshwater environments. Epizootics have been of particular importance in cultured rainbow trout (Salmo gairdneri), ayu (Plecoglossus altivelis), yellowtail (Seriola quinqueradiata), eels and Pacific salmon (Egusa, 1978). It has long been known to cause epizootics among cultured European eels (Anguilla vulgaris) (Anderson and Conroy, 1970) and the disease of cultured salmonids in Europe has more recently been reported (Egidus and Anderson, 1977). First reports of the disease in Pacific salmon held in saltwaters of the Pacific Northwest were by Rucker (1959) and several other reports appeared as the intensity for mariculture of these species increased (Wood, 1968; Cisar and Fryer, 1969; Evelyn, 1971). Vibriosis has been a major problem for maricultured salmonids on the Atlantic coast of Maine and New Hampshire (Sawyer and Strout, 1977) since these operations began in 1972. Without control, the potential for mariculture of many fish species will be limited by this disease.

Immunization of Fish

Historical Perspective

At the turn of this century it was known that fish could mount a specific immunological response to foreign material (Mesnil, 1895; Noguchi, 1903; Metchnikoff, 1905). In the early 1940's Duff (1942) made the first attempts to use this mechanism as a means to control infectious diseases of cultured trout. The following war years interrupted these pursuits and in the 1950's the emphasis for disease control was on antibiotics (Klontz and Anderson, 1970). Renewed interest in the applied aspects of fish immunology began in the 1960's and has since been an active area of investigation.

Parenteral Immunization

Initial efforts to immunize salmonid fish were with bacterins prepared from Aeromonas salmonicida, the etiological agent of furunculosis. Smith (1940) used a heat-killed bacterin to induce specific agglutinin formation in carp (Cyprinus carpio) and rainbow trout after intraperitoneal injection. He showed that at 10°C, an immune response was induced in both species. A formalin-killed preparation from the same bacterin was used by Krantz et al. (1964) to immunize brook trout (Salvelinus fontinalis) and brown trout (Salmo trutta) by intraperitoneal injection. These fish were protected when challenged with live A. salmonicida cells. Spence, Fryer and Pilcher (1965) immunized rainbow trout by intraperitoneal injections

of formalin-killed A. salmonicida cells. A regimen of eight injections was used to produce hyperimmune sera that conferred high levels of immunity in coho salmon receiving serum by passive immunization. A single intraperitoneal injection of A. salmonicida formalin-killed cells emulsified in Freund's Complete Adjuvant was effective for inducing high levels of agglutination titers and protective immunity in coho salmon (Paterson and Fryer, 1974a). The same investigators (1974b) extracted endotoxin from the bacterium and demonstrated its effectiveness for inducing agglutinin formation, also in coho salmon. The endotoxin was not toxic for the fish under the conditions of the experiment.

Concerning V. anguillarum, the disease had historically been associated with cultured eels in Europe and this is where injectable preparations were first tested. European eels were shown (Nybelin, 1935) competent to produce V. anguillarum specific antibodies after several injections of heat-killed cells. Agglutinin formation was temperature related, however, because eels held at 18°C produced antibody and those held at 7°C did not. Muroga and Egusa (1969) obtained similar results with Japanese eels (A. japonica) held at 11 and 15°C. Those held at 11°C did not produce agglutinins while experimental lots immunized at 15, 19, 23 and 27°C had titers after seven weeks. They further showed that the appearance of agglutinins was delayed by two weeks in the 15°C experimental group.

Plaice (Pleuronectes platessa) are another economically important species in which immunization against vibriosis has been studied.

Fletcher and White (1973) compared parenteral and oral methods for induction of antibody production in this species. In the studies pertaining to injection, the killed bacterin was given as a single injection with the bacterial cells suspended in saline or Freund's Complete Adjuvant. After one year, agglutination titers were detectable only in the group injected with adjuvant. Similar findings were reported by Harrell, Etlinger and Hodgins (1975) when serum from parenterally immunized rainbow trout was used to immunize normal trout passively against vibriosis. The titers in fish actively immunized with bacterin emulsified in Freund's Complete Adjuvant were much higher after 62-75 days than in trout receiving a saline-suspended bacterin. Their data also showed that heat-killed cells induced substantially higher titers to V. anguillarum than a formalin-killed preparation. Artificial challenge of trout passively immunized with hyperimmune serum from the injected fish resulted in solid protection against vibriosis when compared to control fish receiving normal trout serum. Rabbit anti-V. anguillarum serum was nearly as effective for conferring protection in passively immunized trout. A further observation by these investigators was that specific antibody began to appear in the serum of passively immunized fish 10 min after injection and reached a peak at 96 h after being administered.

Protection from vibriosis by natural challenge of parenterally immunized coho (Sawyer and Strout, 1977) and sockeye (O. nerka) salmon (Harrell, 1978) has been clearly demonstrated. Sawyer and Strout (1977) showed that cumulative mortality in immunized lots held under

production conditions in a Maine estuary was considerably lower in immunized lots. Also, the percentage of animals reaching minimum market size was significantly greater in the immunized lot.

It was clear that parenterally administered bacterins induced a strong immune response and very high levels of protection against certain fish pathogens. This was proved in many important species of cultured fish. For most fish cultural activities, however, this method is not practical because of the numbers of animals involved. Other mechanisms to immunize fish effectively were needed.

Oral Immunization

Duff (1942) undoubtedly perceived this need when he began work to immunize cutthroat trout orally against furunculosis. Using a diet containing chloroform-killed A. salmonicida cells, protection was demonstrated in bacterin-fed animals exposed to the pathogen by adding it to the water, by holding fish clinically ill with furunculosis together with test lots and by parenteral inoculation. These early observations provided encouragement for future investigators. Klontz and Anderson (1970) reviewed the subject of oral immunization of salmonids and concluded that results to that time were equivocal and further studies were needed. Udey and Fryer (1978) made additional evaluations of an oral A. salmonicida preparation. Their results indicated significant protection of juvenile coho salmon experimentally challenged, however, no significant levels of protection were

observed in production test lots naturally challenged. Few reports concerning furunculosis immunization have appeared since those studies.

Early observations (Fryer, Nelson and Garrison, 1972) concerning the feasibility for orally immunizing Pacific salmon with a V. anguillarum bacterin were promising. A lyophilized sonicate of bacterial cells fed over a 14 day period reduced mortality by about one-half in chinook salmon (O. tshawytscha) naturally challenged in an Oregon estuary. Subsequent studies (Fryer, Rohovec and Garrison, 1978) comparing parenterally and orally immunized coho and chinook salmon, showed that protection of orally immunized fish was nearly as high as that seen in injected fish. They also indicated that specific humoral agglutinins were not detected in orally immunized fish. Similar results had been obtained by Schaperclaus (1972) who immunized carp (Cyprinus carpio) with A. punctata bacterin by the same two routes. Although oral immunization was very promising and many production trials indicated efficacy, higher levels of protection than often obtained by the oral technique were needed. Continued efforts were made to develop new techniques to deliver immunogenic material to fish, particularly salmonids.

Immunization by Immersion

In 1976 (Amend and Fender) a new method for delivering soluble immunogenic material to fish was described. Juvenile rainbow trout were placed in a solution containing sodium chloride (5.23%) at 1650 milliosmoles and 2% of bovine serum albumin (BSA). They were held in

the solution for several minutes and then placed in freshwater at selected one minute intervals. Plasma was collected 45 minutes later and assays for BSA in plasma showed that uptake was linear with time. Levels of 132 µg/ml were detected after a three minute exposure interval. Control fish held in water with no added sodium chloride had less than 10 µg/ml of plasma BSA. Three minutes of hyperosmotic exposure was the maximum that fish could tolerate before mortality began to occur. The technique was given the name hyperosmotic infiltration and the authors proposed its testing for delivery of vaccines and bacterins to fish.

The method was tested (Antipa and Amend, 1977) on pen-reared juvenile coho and chinook salmon infiltrated with V. anguillarum and A. salmonicida bacterins. The infiltrated test lots were compared to intraperitoneally injected groups under conditions of natural challenge in saltwater over a four month period. Agglutination titers for each bacterium were similar as a result of either route of immunization. The natural challenge was primarily by V. anguillarum and after four months, the infiltrated lots had significantly higher survival rates than nonimmunized controls.

Variations of the basic infiltration technique (one-step) were used to immunize sockeye salmon against vibriosis by artificial challenge (Croy and Amend, 1977). The one-step method was modified such that fish were first immersed in the hypertonic solution without bacterin followed by immersion in bacterins with no added sodium chloride (two-step). Various formulations of salt solution were also tested to enhance uptake of antigens. Protection was very high in all

groups infiltrated with undiluted bacterin by the one-step or two-step technique and only subtle differences were noted in test lots exposed to dilutions of the bacterin. Surprisingly, very low agglutinin titers were detected in all groups hyperosmotically infiltrated.

Gould et al. (1978) departed from the concept of using a hypertonic medium for delivery of bacterin. Mechanical force in the form of air pressure was used to simply spray heat or formalin-killed V. anguillarum bacterin on fish and other test lots were immersed for 2 or 60 sec in bacterin without any added salts. Agglutination titers were detected in all test lots and formalin-killed bacterin induced higher titers than the heat-killed preparation. No mortality was observed in any immunized test lot after artificial challenge while nonimmunized controls had a high mortality rate. Their results indicated that exposure of the external surface of the fish body to bacterin was all that was required to induce agglutinating antibodies and high levels of protection. Subsequent studies confirmed that the technique of submerging fish in bacterin for short periods of time was highly effective for inducing solid immunity in fish (Gould, Antipa and Amend, 1979; Egidus and Andersen, 1979; Anderson, Roberson and Dixon, 1979; Kusuda, Kawai and Itami, 1980; Itami and Kusuda, 1980; Viele, Kerstetter and Sullivan, 1980). The method has the added advantage that large numbers of animals can be immunized with a minimum of stress.

The Immune System of Teleost Fish

Phylogenetic Perspective

Fish appear to represent the group of organisms in animal evolution in which integrated cell-mediated and humoral antibody immunity evolved (Cooper, 1976). Fish constitute approximately one-half of the species in the Phylum Vertebrata (Altman and Dittmer, 1972) and therefore the opportunity for development of these complex processes was great. Support for research concerning immune function in this group of vertebrates has been minimal compared to funding for similar mammalian-related studies. Many immunologists, however, have a fundamental interest in lower vertebrate immunological processes and a substantial body of literature has dealt with fish immunology. The phylogeny of immunity was reviewed several times in the last decade and immune processes in fish constitute a large part of this literature (Hildemann and Benedict, 1975; Cooper, 1976; Wright and Cooper, 1976; Marchalonis, 1976; Marchalonis, 1977). The subject of fish immunology was reviewed in 1975 (Corbel) and 1978 (Fletcher). Factors involved in the natural resistance of fish to infection were reviewed by Ingram (1980). An examination of literature pertinent to the studies reported here will be emphasized.

Humoral Immunoglobulins

The immune macroglobulins of three orders of bony fish were characterized by Acton et al. (1971) in an earlier study. The channel

catfish (Ictalurus punctatus) was the representative teleost studied and humoral IgM-like antibodies were described in this species. They were called IgM-like because electron microscopy revealed a tetrameric structure rather than the pentameric structure of IgM found in sharks and higher vertebrates. The purified immunoglobulin had a sedimentation coefficient of 14S and a molecular weight of 610,000. Reduction and alkylation yielded heavy (H) and light (L) chains with molecular weights of 70,000 and 23,000 daltons, respectively. The amino acid composition of these chains were similar to those of mammalian IgM.

Coho salmon humoral antibodies specific for A. salmonicida whole cells were characterized by Cisar and Fryer (1974). Serum was collected 12 weeks after parenteral immunization and the antibody was purified by dissociation of cell wall agglutinates and gel filtration. It eluted in the first peak from a Sephadex G-200 gel filtration column and had a tetrameric structure analogous to the channel catfish antibody. A 17S sedimentation coefficient was determined and reduction and alkylation produced H and L chains of approximately 75,000 and 26,000 daltons, respectively. The molecular weight of monomeric subunits was estimated at 189,000 daltons.

Monomeric and tetrameric immunoglobulins were described (Clem and McLean, 1975) in the margate (Haemulon album), a marine teleost, after parenteral immunization with BSA. Sedimentation coefficients of 16 and 7S were determined for the tetramer and monomer, respectively. They were antigenically identical and the monomer was detectable only

by antigen-binding assays. It was not revealed by a less sensitive hemagglutination assay as was the tetramer. The 7S monomer eluted in the second peak from a Sephadex G-200 molecular sieve column.

Results not unlike Cisar and Fryer's (1974) were obtained by Ingram and Alexander (1979) with brown trout immunoglobulin and Clerx et al. (1980) with pike (Esox lucius) immunoglobulin. Evidence was obtained for tetrameric antibodies with components having properties similar to those of the coho antibody. No evidence of non-tetrameric forms was given in either report.

O'Leary (1980) identified high molecular weight (HMWIg), 620,000 daltons, and low molecular weight (LMWIg), 490,000 daltons, immunoglobulin populations in the serum of rainbow trout immunized with a trinitrophenol hapten. The H chain (72,000 daltons) from each population had an identical molecular weight as did the L chains (27,000 daltons) determined by SDS-polyacrylamide gel electrophoresis. While no data was given on the relative concentrations of each population in serum, the results indicate that they were similar.

Lobb and Clem (1981) also demonstrated the presence of HMWIg (∿700,000 daltons) and LMWIg (∿350,000 daltons) populations in the serum of nonimmunized sheepshead (Archosargus probatocephalus). They further separated the HMWIg into two subpopulations (11 and 14S entities) by analytical ultracentrifugation in a denaturing solvent. Antigenically they were identical, and the H chains of each HMWIg had the same molecular weight (70,000 daltons) as did the L chains (25,000 daltons). The LMWIg had heavy and light chain weights of

45,000 and 25,000 daltons, respectively. Immunoelectrophoresis of whole sheepshead serum against rabbit anti-HMWIg serum failed to reveal the presence of the LMWIg even though it was antigenically related. The authors attributed the failure to detect the LMWIg protein by this method to the fact that it was present in serum at 1/20th the concentration of the HMWIg. These results indicate that a similar level of immunoglobulin complexity potentially exists in other teleosts. This complexity has only recently been possible to realize with the development of increasingly sensitive assays.

Secretory Immunoglobulins and Localized Immunity

All vertebrates are known to possess accumulations of lymphoid tissue in the gastrointestinal tract (Cohen, 1975) and lymphocytes that migrate through the intestinal epithelium have been described in vertebrates phylogenetically as primitive as anuran amphibians (Goldstine, Manickavel and Cohen, 1975). These tissues and cells are effectors of localized immunity with cellular and secretory components.

Gut-associated antibody in plaice orally immunized with V. anguillarum for one year was described by Fletcher and White (1973). Antibody titers in intestinal mucus were higher than serum agglutinin titers in fish orally immunized. The converse was observed in fish parenterally immunized.

A recent study (Lobb, 1980) should provide renewed interest in the area of extra-humoral immunoglobulins in fish. Two secretory

immunoglobulins were isolated from sheepshead bile and cutaneous mucus differing from the three humoral immunoglobulins that Lobb and Clem (1981) described. The bile immunoglobulin was dimeric with a molecular weight of approximately 320,000 daltons. A dimeric structure was also reported for the mucus protein that had an estimated molecular weight of 350,000 daltons. The H chains of these and the serum immunoglobulins were antigenically similar and he postulated that all of the antibodies were of the same class. Radiolabeled serum immunoglobulins were assayed in serum, bile and mucus after intravenous injection and it was shown that the secretory antibodies could not have been derived by transudation of serum antibodies. This study should set a precedent for future studies in other fish species.

Temperature, Infection and Immunity in Fish

Reference has already been made (Nybelin, 1935; Muroga and Egusa, 1969) concerning the repressive effect of lowered environmental temperature on immune processes in fish. Meyer (1970) examined the impact of seasonal fluctuations on disease outbreaks in fish farms and concluded that April was the month in which the greatest number of disease outbreaks occurred. Snieszko (1954) had previously hypothesized that differences between metabolic rates of pathogens and their hosts during periods of fluctuating water temperatures could explain these kinds of observations. An in-depth examination (Avtalion et al., 1973) of the influence of environmental temperature on the immune response in fish complemented these earlier reviews.

Investigations dealing with the effects of water temperature on infection and the immune response in salmonid fish were reported by Fryer and Pilcher (1974) and Fryer et al. (1976). Many of the major bacterial pathogens of Pacific salmon were used to infect and produce immune responses in fish held at selected temperatures. With the notable exception of bacterial kidney disease, a higher mortality and a shorter mean day to death was observed in experimental lots infected and held at the higher temperatures. Favorable temperatures for the progress of infection with bacterial kidney disease were in the range of 6.7 to 12.2°C. Experimental lots immunized and held at the higher temperatures tended to respond quicker and mounted a more vigorous response than those held at lower temperatures. In a later

study, (Hetrick, Fryer and Knittel, 1979) this parameter of infection was tested in rainbow trout exposed to infectious hematopoietic necrosis virus at seven water temperatures. With this viral disease, mortality was higher in experimental lots held at elevated temperatures.

MATERIALS AND METHODS

Animal Passage and Preservation of a *Vibrio anguillarum* Isolate

A culture of *V. anguillarum* (LS 174, Serotype I) on a Cytophaga Sea Water Agar deep was provided by Dr. J. S. Rohovec of this laboratory. The organism was first isolated from morbid juvenile chinook salmon undergoing a natural epizootic of vibriosis at Lint Slough, Waldport, Oregon in July, 1974.

To ensure virulence of the organism it was cultured in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Michigan) at 18°C with aeration and this broth culture was added to 20 l of 18°C aquarium water containing ten juvenile coho salmon (mean weight 35 g/fish). Bacterial cultures from kidney tissue of fish that died were grown and used to infect a second group of fish. This procedure was carried out so that the original isolate was passed three times by water-borne exposure in fish.

Colonies obtained in pure culture from the third group infected were identified as *V. anguillarum* (Serotype I) by 1) colony morphology; 2) cell motility, size, shape and Gram reaction; 3) sensitivity to novobiocin and 0/129 (2,4-diamino-6,7-di-iso-propyl pteridine); and 4) agglutination with specific rabbit antiserum. Cultures were grown in broth, washed three times in sterile saline (0.85% NaCl), resuspended in fetal bovine serum (Flow Laboratories, McLean, Virginia) and lyophilized.

Experimental Animals

Fish Husbandry

With the exception of one set of experiments, the coho salmon stock used throughout this study was propagated at the Fall Creek Salmon Hatchery, Oregon Department of Fish and Wildlife (ODFW). The Bonneville Salmon Hatchery (ODFW) stock was used for the experiments concerning the effect of water temperature on mortality of juvenile coho salmon experimentally infected with V. anguillarum.

Animals were transported to the Oregon State University Fish Disease Laboratory, Corvallis, Oregon (OSU FDL) in aerated containers. Water temperature was maintained with the addition of ice when necessary. Eggs were transported in plastic beakers on ice. Fish and eggs were held in aquaria continuously supplied with fish pathogen-free 12°C well water. Various sizes of aquaria were utilized to minimize loading density. Oregon Moist Pellet (OMP) ration was fed ad libitum daily.

General Techniques Applied to Fish

Immobilization of fish for injection, bleeding, branding or individual immersion was attained by anesthetization in a solution of benzocaine (50 mg/1). For injection and branding, animals were anesthetized to Stage III, Plane I while for bleeding and individual immersion they were anesthetized to Stage III, Plane III (McFarland and Klontz, 1969).

Injections were given with 1 ml tuberculin syringes (23 or 26 gauge needles). Intravenous injections were through the duct of Cuvier (Sano, 1960) as were all bleedings (1 ml tuberculin syringe, 26 gauge needle) of fish for which repeated collection of serum was necessary. Blood was collected from fish sacrificed at the time of bleeding by severing the caudal fin and allowing blood to flow into centrifuge or capillary tubes.

Identification of fish from different treatment groups in the same aquarium was accomplished by cold-branding with a probe immersed in a dry ice-acetone bath. Brands appeared 2-3 days after branding and were retained for about 30 days.

Bacterins

Injectable Bacterin

For immunizing fish by injection (parenterally), preparations of killed cells administered intraperitoneally were used. Saline washed V. anguillarum cells were resuspended in sterile phosphate buffered saline (PBS), pH 7.2, to which a 0.3% volume of formalin was added. The cells were pelleted by centrifugation after being held 12 h at 4°C. The pellet was resuspended in sterile PBS at a volume to give the desired cell concentration for injection. Sterility of the bacterin was determined by inoculating a BHI agar plate with 0.1 ml of the cell suspension. The bacterin was aliquoted into sterile, rubber-stoppered serum bottles from which syringes were filled for animal injections.

Oral Bacterin

Fish that were immunized orally were fed a bacterin-containing diet. The bacterin preparation consisted of mass cultured cells produced from an isolate of V. anguillarum (LS 173) biochemically and serologically identical to LS 174 (J. S. Rohovec, personal communication). Frozen, wet-packed cells were sent to the Oregon State University Seafoods Laboratory, Astoria, Oregon where they were incorporated into OMP diet at a level of 5 mg wet weight of packed cells per gram of OMP. Based on triplicate dry weight determinations of the wet-packed cells this provided a diet containing about 1 mg of

cellular material per gram of diet. This level was previously shown to provide protection from vibriosis in fish fed over a 15 day period (Rohovec, 1974). Pelletized OMP appropriate for the sizes of fish receiving the ration were kept frozen at -20°C until fed.

Immersion Bacterin

A suspension of late stationary growth phase cells was utilized for immunizing fish by immersion. Four liters of BHI broth were inoculated with 100 ml of V. anguillarum grown from the lyophilized stock. Incubation for five days at 18°C with aeration allowed maximum growth and cell lysis. Microscopic examination after five days indicated that only five to ten percent of the cells appeared morphologically intact and motile. Purity was determined by agglutination with rabbit antiserum specific for V. anguillarum and by streaking BHI agar plates. Formalin (0.3%) was added to kill the cells and incubation continued for 24 h. The pH was adjusted to 7.0 with 2.0 N NaOH and sterility determined. One liter volumes of the bacterin were aliquoted into plastic bottles and frozen at -20°C. Triplicate dry weight assays of this preparation indicated that it contained a bacterial mass of 4.0 mg/l.

Challenge Method

The method described by Gould et al. (1978) for water-borne infection of fish with V. anguillarum was followed. Pure cultures were grown at 18°C from lyophilized stocks. Approximate cell numbers were estimated by optical density measurements of the culture at 525 nm. Triplicate plate counts were then made to more accurately enumerate viable cells in the challenge medium. These broth cultures were mixed into aquarium water containing the fish and a constant exposure dose (10^5-10^7 cells/ml) was maintained for 15 min. After this exposure, water flow was restored to normal levels resulting in a gradual dilution of the bacterial cells from the aquarium.

Infected fish that died from this method of exposure usually did so between two and eight days. Therefore all experiments to induce infection were terminated ten days after exposure, with the exception of the experiment concerning the effects of temperature on mortality.

Kidney tissue smears from all morbid fish were made on BHI agar plates. Isolates were identified by colony morphology and sensitivity to novobiocin and 0/129. Twenty percent of the isolates were further confirmed to be V. anguillarum by agglutination with specific antiserum.

Comparison of Methods for Immunizing Fish

Intraperitoneal Absorption of Tetrameric Immunoglobulin and Reduced Monomer after Parenteral Administration

The potential for specific antibody to protect animals given the immunoglobulin (passive immunity) but not the immunogen depends on the uptake and distribution of the protein after administration. A quantitative measure of the uptake of radiolabeled purified tetrameric immunoglobulin and its dissociated monomer in intraperitoneally injected coho salmon was made. The intraperitoneal absorption kinetics of monomeric subunits of the tetramer were compared to the intravenous metabolism of the monomer in a second experiment. The methods were similar to those described for the tetramer experiment.

Serum from nonimmunized juvenile coho salmon (mean weight 33 g/fish) was precipitated for 24 h with an equal volume of saturated ammonium sulfate at 4°C. After dissolution in PBS (pH 7.4) the ammonium sulfate precipitation was repeated and the precipitate dissolved in and extensively dialyzed against 0.05 M phosphate buffer, pH 7.5. The protein solution was clarified by centrifugation (13,000 x g, 10 min, 0°C) and applied to a DEAE-cellulose ion exchange column (2 x 10 cm) equilibrated in 0.05 M phosphate buffer, pH 7.5. The immunoglobulin fraction was eluted under these conditions of pH and ionic strength. Additional purification was attained by molecular sieve chromatography over a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (2 x 50 cm) equilibrated in PBS. Purity was assayed by immunoelectrophoresis against rabbit antiserum specific for coho whole serum.

The purified immunoglobulin was radioiodinated with $Na^{125}I$ using the chloramine-T oxidation method (Sonada and Schlamowitz, 1970). Labeled protein was dialyzed against PBS at 4°C to remove unconjugated ^{125}I and conjugation was confirmed by precipitability of labeled protein (>95%) in 5% trichloroacetic acid as measured on a gamma counter (Packard Auto-Gamma spectrometer, Series 410A). Immunoglobulin concentration was calculated on the basis of an extinction coefficient ($E^{1\%}_{278\ nm}$) of 16 (Cisar and Fryer, 1974) and measuring ultraviolet light absorption at 278 nm (Perkin Elmer double beam spectrophotometer). A specific activity of labeled immunoglobulin of approximately $1\text{-}2 \times 10^7$ cpm/mg protein was obtained.

A portion of the purified coho salmon tetrameric immunoglobulin was reduced to monomeric subunits by incubation at room temperature for 2 h with 0.01 M 2-mercaptoethanol (Voss et al., 1980). Ten molar excess of iodoacetamide was added to alkylate sulfhydryl groups on monomers thus preventing reassociation to tetramers. Monomers were eluted as a peak by passage through a Sephadex G-200 column equilibrated in PBS.

A 0.05 ml volume of radiolabeled tetrameric immunoglobulin was injected intraperitoneally into each of two coho salmon (one yearling and one two-year old) and intravenously, through the duct of Cuvier, into two other fish of the same year classes. Duplicate 0.1 ml blood samples from each fish were taken at selected intervals from 4 to 96 h after injection and counted in the gamma counter. Larger animals were required for these experiments because of the volume of blood required.

Protection of Fish Passively Immunized with Partially Purified Anti-*Vibrio anguillarum* Immunoglobulin

Coho salmon immunoglobulin purified by chemical and chromatographic techniques was injected into fish to demonstrate its capacity to provide protection in vivo. Techniques similar to those used to purify tetrameric immunoglobulin for the absorption studies were employed to partially purify immunoglobulin from juvenile coho salmon parenterally immunized against *V. anguillarum*. Serum was obtained from juvenile coho salmon (mean weight 30 g/fish) 60 days following injection of 0.1 ml *V. anguillarum* bacterin containing 10^{10} cells/ml. The pooled serum was precipitated once with ammonium sulfate as previously described. The precipitate was then dissolved in and dialyzed extensively against 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl at 4°C. The antibody containing suspension was concentrated from approximately 15 to 2 ml by pressure dialysis over an XM100 membrane (Amicon, Lexington, Mass.) and applied to a Sephacryl S-300 (Pharmacia) gel filtration column (2.6 x 60 cm) equilibrated in the Tris-HCl buffer. Fractions of 2.5 ml were collected and assayed for protein content by ultraviolet absorption at 280 nm using a Beckman Model 35 spectrophotometer (Beckman Instrument Co., Irvine, Calif.).

A curve was plotted from OD_{280} measurements and *V. anguillarum* antibody titers were performed on selected fractions. The fraction from the first peak off the column (0.12 $OD_{280\ nm}$) with a 1:4 agglutination titer was injected intraperitoneally into 50 juvenile coho salmon (0.05 ml/fish) having a mean weight of 2.8 g/fish. The

animals were distributed equally into two aquaria (12°C) as were two 25-fish control groups injected with (0.05 ml/fish) column buffer. The four groups of fish were exposed to 1.1×10^6 V. anguillarum cells/ml of aquarium water by water-borne challenge.

Protection of Fish Administered Serum or Spleen Cells from Parenterally or Orally Immunized Donors

The failure of several investigators to detect significant levels of agglutinating antibody in the serum of fish orally immunized against V. anguillarum suggested a mechanism of protection independent of circulating antibody (Fletcher and White, 1973; Rohovec, 1974). To determine if a nonagglutinating serum immunoglobulin component or cellular immune response was induced as a result of oral immunization, whole serum and spleen cells from actively immunized (received immunogen) donors were injected into normal recipients (received no immunogen) which were subsequently challenged. A parenterally immunized and a nonimmunized group served as positive and negative controls, respectively (Figure 1).

Five-hundred fifty juvenile coho salmon (mean weight 14 g/fish) in each actively immunized and control treatment group were divided into two, three-foot circular fiberglass aquaria. Each parenterally immunized fish received a single 0.1 ml intraperitoneal injection of suspended V. anguillarum bacterin containing 2.6×10^9 cells/ml. Orally immunized animals were fed the bacterin in OMP for 15 consecutive days at 2% body weight of diet per day. Fish in the control group received a single 0.1 ml intraperitoneal injection of sterile PBS.

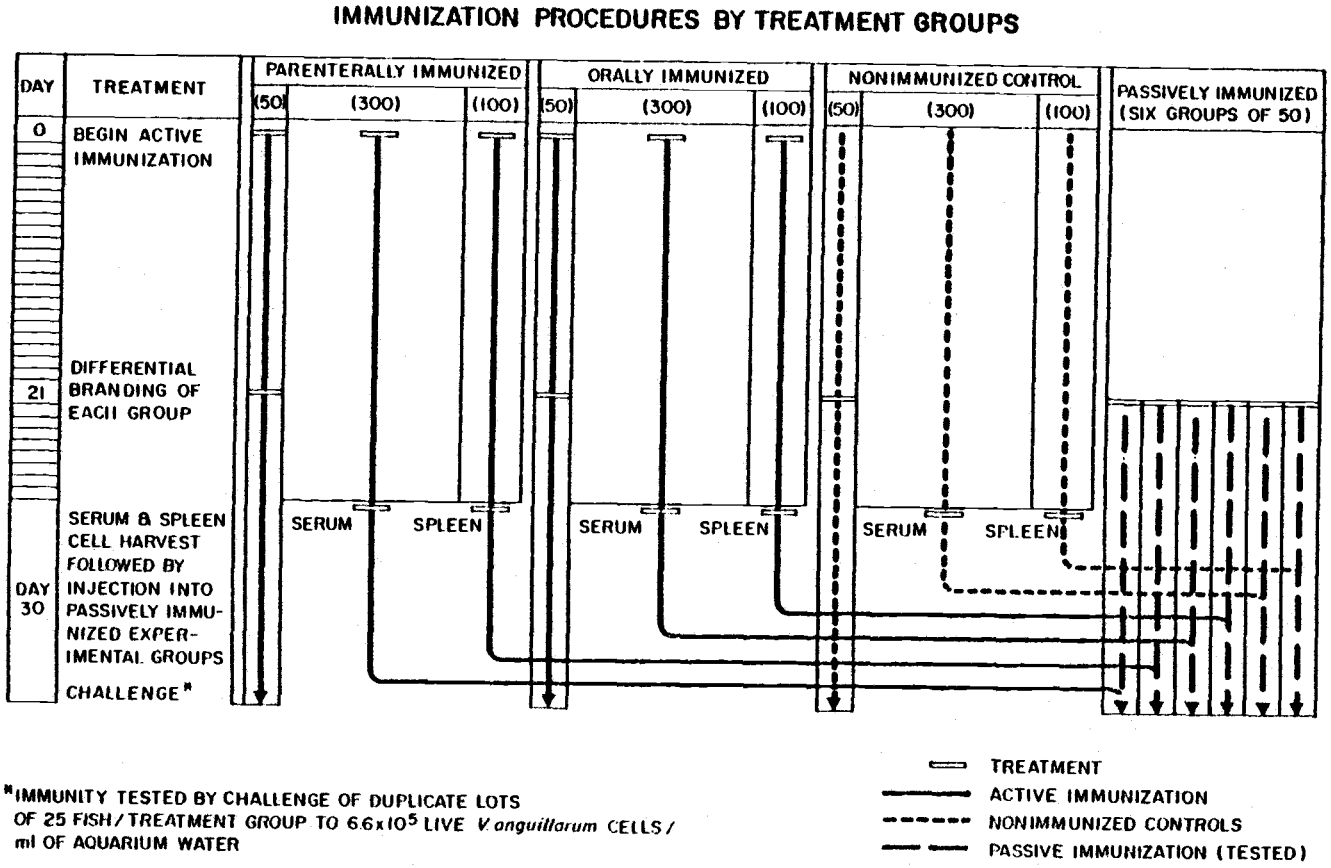


Figure 1. Flow diagram of experimental design to test protection of fish injected with serum or spleen cells from parenterally or orally immunized fish. Numbers in parenthesis below treatment group heading represent the number of fish given the indicated treatments.

Twenty-one days after immunization began, 50 fish from each treatment group and six groups of 50 normal fish were differentially cold-branded and placed in separate aquaria.

Thirty days post-immunization the sera from 300 and spleens from 100 of the remaining 500 fish in each group were taken. Blood was collected in sterile centrifuge tubes and whole spleens were removed and placed in tubes (five spleens/tube) containing 2.0 ml of sterile Hanks Balanced Salt Solution (Flow Labs, Rockville, MD) (HBSS), pH 7.2 on ice. Serum was harvested, filtered through a 0.45 µm filter (Millipore Corp., Bedford, Mass.) into sterile serum bottles and aspirated into 10 ml disposable syringes. A microtiter assay of each serum pool was made. Tubes of whole spleens were macerated with a tissue grinder (Tri-R Instruments, Rockville Center, New York) and filtered through four layers of sterile cheesecloth. The pooled spleen cells from each group were washed two times in 100 ml HBSS by centrifugation (1,000 x g, 10 min, 0°C) and resuspended in 50 ml HBSS (100 spleens/50 ml). Duplicate cell counts from each spleen cell pool were made with a hemocytometer. Disposable syringes (10 ml) were filled with the cell suspension having a cell concentration equivalent to one spleen/0.5 ml.

Each of the six groups of 50 untreated fish previously cold-branded, were intraperitoneally injected with 0.5 ml of serum or spleen cells from immunized (orally or parenterally) or nonimmunized donors. The 50 actively immunized and control animals were anesthetized and punctured with a 26 gauge needle to duplicate the trauma which the

passively treated fish received. Duplicate lots of 25 fish from each group were separated after handling into two 114 l rectangular aquaria (18°C). Fish in each aquarium were exposed to 6.6×10^5 V. anguillarum cells/ml of aquarium water by water-borne challenge. Smears from kidney tissues of all fish that died and each survivor were made on BHI agar plates that were then examined for V. anguillarum.

Immunity Induced by Oral or Water-borne Exposure of Fish to Bacterin

Immersion and spray methods are effective techniques for immunizing fish (Antipa and Amend, 1977; Gould et al., 1978; Gould et al., 1979). In these procedures the external surface of the animal is briefly exposed to immunogenic material. An experiment was designed to determine if oral immunization is actually a manifestation of the immune response induced by immersion or spray immunization.

After cold-branding, 60 juvenile coho salmon (mean weight 4.5 g/fish) were placed into each of three 68 l aquaria (15°C). Fish in one aquarium received OMP at an amount equal to 2% body weight/day for 30 days (control). A second group was given an identical amount of OMP containing V. anguillarum bacterin (5 mg/g of diet) for 15 consecutive days followed by normal OMP diet for 15 days (orally immunized). The third group was fed OMP at a similar level for 30 days; however, for the initial 15 days, 27 mg/day wet weight of the same V. anguillarum whole cell bacterin used to make the oral preparation was mixed into the aquarium water (water-borne exposure). This amount of bacterin corresponded to that incorporated into the daily ration of

the orally immunized group. It was mixed into the water 15-30 min after the fish were fed each day.

After 30 days, 50 fish from each treatment group were separated into two aquaria (18°C) and challenged by the water-borne method with 5.0×10^6 V. anguillarum cells/ml of aquarium water. The ten remaining fish from each group were bled and individual serum antibody titers determined.

Immunity Induced by Immersion of Selected Portions of the Fish Body in Bacterin

An experiment was designed to determine if immersing selected portions of the animal's body led to different levels of protection. Important sites for uptake of antigen could be postulated if significant differences were observed.

A 100 ml aliquot of immersion bacterin was diluted 1:10 (0.4 mg/ml) in 12°C aquarium water. Six groups of 60 juvenile coho salmon (mean weight 10.7 g/fish) were anesthetized and portions of their bodies immersed in undiluted (4.0 mg/ml) or 1:10 diluted V. anguillarum bacterin for 1 min. A seventh group was completely immersed in undiluted bacterin without anesthetization and the eighth group was only anesthetized. The body areas immersed were: 1) head only; 2) posterior body, excluding the head and 3) the entire fish. The fish in the first group were individually held in the bacterin such that the head, anterior to approximately where the operculum attaches to the dorsal side of the body, was immersed. Opercular movement had begun by the time the 1 min interval had ended and therefore bacterin was pumped

over the gills during the latter part of the immersion period. The fish in the second group were individually held so that the posterior portion of the body from about 1 cm anterior to the dorsal fin was immersed. The third group was totally submerged in the suspension. To prevent carryover of bacterin to aquaria, fish were rinsed three times after immersion in containers of 12°C water.

Thirty days post-immersion 50 fish from each group were cold-branded and divided equally into two 68 l aquaria (18°C). They were challenged with 2.0×10^5 V. anguillarum cells/ml of aquarium water by the water-borne method. The ten remaining fish from each treatment group were bled and individual antibody titrations performed.

Exposure of Eggs to Bacterin During Artificial Spawning

Immersion offers a potential method whereby fish eggs may be safely exposed to immunogenic material. Evidence for an immune response in juveniles derived from eggs exposed to bacterin during artificial spawning was evaluated.

Coho salmon eggs from seven adult females at the Fall Creek Salmon Hatchery, ODFW, were separated into three groups. The eggs were collected in dry plastic beakers and 5 ml of V. anguillarum immersion bacterin, temperature equilibrated to hatchery water temperature (4.2°C), was added to one group before fertilization (fertilized in bacterin group). The milt from four males was pooled and a 1 ml aliquot was stirred into each beaker of eggs. Fifteen minutes later the eggs were washed three times. After the third wash,

500 ml of hatchery water was added to each beaker along with 5 ml of bacterin to the second treatment group (water-hardened in bacterin group) and a third group was untreated (control). Water-hardening was allowed to continue for 1 h after which the eggs were washed three times again, covered with water and transported on ice to the OSU FDL. The eggs were placed in separate aquaria and not handled further except for the removal of dead eggs when observed.

After hatching the young fish were periodically challenged with live V. anguillarum. At a mean weight of 0.67 g/fish, 50 alevins (Hubbs, 1943) from each of the three egg treatment groups were separated into five aquaria and tempered to 18°C. An additional ten fish from each of the groups were placed together in an aquarium (18°C) and held as nonchallenged controls. Twenty-four hours later, one aliquot from each of five successive ten-fold dilutions of live V. anguillarum was added to an aquarium of each group. This provided exposure concentrations ranging from 2.0×10^3 to 2.0×10^7 cells/ml. In these experiments, fish from the different treatment groups and challenged with the various concentrations of V. anguillarum could not be held in a common aquarium because they were too small to cold-brand and later identify. When the coho reached mean weights of 0.75 and 0.86 g/fish similar procedures were again followed. The two lower exposure concentrations were eliminated because no mortality attributable to V. anguillarum was anticipated at these levels based on results from the exposures at 0.67 g. The exposure dose corresponding to 2.0×10^7

cells/ml in the first experiment (0.67 g/fish) was 2.3×10^7 cells/ml at 0.75 and 4.0×10^7 cells/ml in the 0.86 g fish.

When the remaining fish derived from the original egg treatment groups attained a mean weight of 1.2 g/fish they were tested for immunological tolerance. Fifty fish from each lot were cold-branded and intraperitoneally injected with 0.05 ml of bacterin (1.7×10^9 cells/ml). The same number of noninjected fish from each group were also cold-branded. Thirty days later 40 fish (mean weight 3.3 g/fish) from each lot were evenly distributed into two aquaria (18°C) and challenged by the water-borne method with 1.0×10^7 V. anguillarum cells/ml of aquarium water.

Exposure of Embryonated Eggs to Bacterin by Injection or Immersion

Lymphoid tissues in salmonid fish embryos have recently been described (Ellis, 1977). With this knowledge and to complement the studies with eggs, an experiment was designed to test immunological competence of embryos exposed to V. anguillarum bacterin by immersion or injection. Coho salmon eggs from ten females fertilized with milt from five males were collected at the Fall Creek Salmon Hatchery (ODFW) and reared at the OSU FDL. When the embryos were estimated to be within 7 days of hatching they were exposed to V. anguillarum bacterin as follows: 1) the immersed group was placed in temperature equilibrated immersion bacterin for 2 min; 2) fifty were injected in the yolk material with 0.01-0.02 ml of V. anguillarum bacterin containing 10^{10} cells/ml; and 3) the control group received no treatment. The embryos

in each group began hatching 4 days post-treatment and had completed emergence by day 5. Each of the groups was reared in separate aquaria. Only six fish from the injected group survived until the initial bleeding 218 days later.

Two-hundred three days post-hatching six fish from the control group and seven from the immersed group were randomly selected and cold-branded as were the six from the injected embryo group. They were held in separate aquaria and on day 218 post-hatching were bled, intraperitoneally injected with 0.1 ml V. anguillarum bacterin containing 5×10^9 cells/ml and returned to their aquaria. Six fish from the original control embryo group were also bled and injected with PBS as controls. Thereafter, individual fish were bled every fourth day for 28 days and the serum harvested and frozen. Vibrio anguillarum agglutination titers were determined after the last day of sampling.

Effects of Temperature on Immunity and Infection

Growth Rate of *Vibrio anguillarum* at Selected Temperatures

The growth rate of the bacterium in broth medium at three temperatures was measured. Log phase *V. anguillarum* cells growing in BHI broth at 12°C were used as inoculum (0.5 ml) for three side-arm flasks each containing 50 ml of BHI broth (0.02% antifoam) equilibrated to 6, 12 and 18°C. Aeration was attained by sparging sterile air into the culture medium. The flasks were maintained at each temperature and optical density measurements at 520 nm recorded at selected intervals throughout the growth period.

Effect of Temperature on the Onset of Serum Agglutinin Formation

Temperature as a parameter in controlling the onset of specific antibody formation in fish was examined. Juvenile coho salmon (mean weight 11 g/fish) were tempered to each of three water temperatures: 6, 12 and 18°C. After acclimation for ten days at these temperatures one group at each temperature was parenterally immunized with 0.1 ml *V. anguillarum* bacterin containing 5 mg dry weight of cells/ml. The second group at each temperature was injected with 0.1 ml of PBS. Five fish from each group were sacrificed and bled at five day intervals for the next 30 days. Sera were frozen at -70°C after harvest and individual *V. anguillarum* antibody titrations determined.

Effect of Water Temperature on Mortality of Experimentally Infected Juvenile Coho Salmon

The relationship of water temperature and mortality of fish experimentally infected with V. anguillarum by water-borne exposure at selected temperatures was examined. Duplicate groups of 25 juvenile coho salmon (Bonneville Salmon Hatchery) with a mean weight of 19 g/fish were tested at seven water temperatures ranging from 3.0 to 21.0 in increments of 3.0°C. Tempering fish to experimental temperatures was by the method of Holt et al. (1975). Groups were exposed to 2.0×10^6 cells/ml by the water-borne challenge method in 68 l aquaria. Two unexposed control groups at each temperature were treated identically except no V. anguillarum cells were added to the water. The experiment continued for 30 days with all morbid and surviving fish having kidney smears made on BHI culture medium.

Intraperitoneal Absorption Kinetics of Tetrameric Immunoglobulin at Selected Temperatures

Absorption of radiolabeled tetrameric immunoglobulin from the peritoneal cavity was followed at 6, 12 and 18°C. Materials and methods were identical to those used in the tetramer and monomer absorption studies except that fish were tempered for 48 h to the three water temperatures before receiving labeled tetramer either intravenously or intraperitoneally.

Serological and Immunological Methods

Production of Rabbit Antisera

Rabbit antisera was produced using bacterial and protein antigens emulsified in Freund's Complete Adjuvant (Difco Laboratories, Detroit, Mich.). Injections were given in the hind footpads (0.3 ml/foodpad) and subcutaneously (1-2 ml) above the shoulders in New Zealand white rabbits. Blood was collected from the marginal ear vein 12 or more days post-immunization. Clot retraction was allowed to proceed for 2 h at room temperature and then at least 8 h at 4°C. The serum fraction was collected after centrifugation (0°C, 10 min, 3,000 x g) and frozen at -20 or -70°C in sterile vials or tubes (1-5 ml).

Harvesting Fish Sera

Individual or pooled fish blood samples for serological assays were collected in centrifuge tubes. Clot retraction was allowed for 1 h at room temperature then at least 8 h at 4°C. The serum fraction was clarified by centrifugation, harvested and stored as described for rabbit sera.

Harvesting Fish Body Mucus

External body mucus for immunological assays was absorbed from anesthetized fish with cotton swabs. Mucus was expressed from the swabs, pooled and centrifuged (0°C, 10 min, 10,000 x g). The super-

natant was concentrated ten-fold by pressure dialysis over an XM100 (Amicon) membrane at 4°C and stored as described for rabbit sera.

Microtiter Assay of Agglutinating Antibody in Fish Serum

Serum agglutination titers against V. anguillarum were determined using the microtiter technique. Depending on the serum volume available for assay, either the 25 or 50 µl system (Cooke Engineering, Alexandria, Virginia) was used. Antigen was live V. anguillarum whole cells washed three times in PBS and adjusted to an optical density of 0.85 at 525 nm. Titers were determined after incubation of plates for 2 h at room temperature followed by 12 h at 4°C.

Immunoelectrophoresis

Immunoelectrophoretic methods for detecting serum and mucus macroglobulins were conducted on a Gelman Deluxe Electrophoresis Chamber (Gelman Instrument Co., Ann Arbor, Mich.). Barbital buffer (pH 8.6) was used as chamber buffer and diluent for 1% agarose (Marine Colloids, Inc., Rockland, Maine) gels. Samples were electrophoresed at 2.5 ma/slide (25 x 75 mm) for 2 h. Rabbit antiserum was added to the trough and immunodiffusion allowed to proceed at 4°C until precipitin bands developed.

Purification of Juvenile Coho Salmon Serum Immunoglobulin

Affinity and molecular sieve chromatographic methods (O'Leary, 1980) were employed to purify coho salmon serum antibody specific

for a trinitrophenol (TNP) hapten. Trinitrophenol hapten was covalently bound to keyhole limpet hemocyanin carrier, emulsified in Freund's Complete Adjuvant and intraperitoneally injected (0.1 ml/fish) into juvenile coho salmon (mean weight 38 g/fish). The fish were then bled three times between 30 and 60 days after immunization.

The harvested serum (25 ml) was subjected to lipid extraction, 50% ammonium sulfate precipitation and dialysis against 0.1 M phosphate buffer, pH 8.0. Dialyzed protein was mixed for 20 h at 4°C with an affinity matrix designed to bind TNP specific antibodies. The affinity matrix consisted of Sepharose 6B (Pharmacia) beads to which a ligand, bovine serum albumin conjugated with TNP, had been covalently bound (March, Parikh and Cuatrecasus, 1974). After absorption, the affinity matrix was extensively washed and bound coho anti-TNP antibody was eluted with 3.0 M KSCN. Eluted protein was dialyzed against 0.1 M phosphate buffer to remove KSCN, concentrated by pressure dialysis and applied to a Sephacryl S-300 gel filtration column (2.6 x 60 cm). Immunoelectrophoresis against rabbit antiserum specific for coho whole serum and electrophoresis in a sodium dodecyl sulfate polyacrylamide gel were used to further determine purity of the protein peak from the S-300 column. Electrophoresis in the gel (10% acrylamide) was on a vertical slab electrophoresis cell (Bio-Rad model 220, Bio-Rad Labs, Richmond, CA) run at 6 ma for 19 h. Low molecular weight standards (Bio-Rad) were run next to the immunoglobulin sample and the protein bands were stained with Coomassie Blue.

Production of Rabbit Antiserum Specific for Coho Salmon Serum Immunoglobulin

Purified juvenile coho salmon anti-TNP immunoglobulin was used to produce rabbit antiserum specific for the coho salmon serum immunoglobulin. The coho salmon serum anti-TNP fraction (1.5 ml) collected from the peak of the Sephacryl S-300 column was injected into a rabbit after purity was determined. The fraction had an absorbancy of 0.3 at 280 nm. Collection of rabbit serum began 15 days later and its purity established by precipitation with electrophoresed coho whole serum.

Immunoelectrophoretic Assay for Immunoglobulin in Fish Mucus

Several attempts were made to measure agglutinating antibody in the mucus of fish that had been immunized by immersion and had serum titers as high as 1:8192 against V. anguillarum. Weak titers of 1:4 were the highest that could be detected in mucus, although no agglutination could be observed at a 1:4 dilution of mucus from nonimmunized fish. Therefore, immunoelectrophoretic methods were used to assay for the presence of immunoglobulin in coho salmon mucus. Rabbit antiserum specific for coho serum immunoglobulin was added to the troughs of immunoelectrophoretic gels and allowed to diffuse against electrophoresed mucus proteins.

RESULTS

Comparison of Methods for Immunizing Fish

Intraperitoneal Absorption of Tetrameric Immunoglobulin and Reduced Monomer after Parenteral Administration

Comparative counts in the blood between fish injected intravenously and intraperitoneally with radiolabeled purified tetrameric coho salmon immunoglobulin are shown in Figure 2. Two fish received labeled protein by each route of injection and when the cpm in the blood were normalized to a standard body weight of 100 g, they were quantitatively similar for each treatment. The experiment yielded three results. First, absorption of the immunoglobulin from the peritoneal cavity was relatively rapid and was complete in 8 h. Similarly, equilibration of intravenously administered immunoglobulin with the blood and extravascular tissues was reached by 8 h. Second, after 8 h the amount of labeled material absorbed from the peritoneal cavity was approximately 60% of the total labeled protein present in the blood of intravenously injected fish. Finally, the slopes of the four lines (Figure 2) show that the rates of catabolism after 8 h were similar and the calculated half-life values were 46 and 52 h for intraperitoneal and intravenous injection, respectively.

The monomer obtained by thiol reduction of the tetramer was less effectively absorbed when compared to its equilibration after intravenous injection. Figure 3 represents a plot of comparative rates of absorption (intraperitoneal), tissue equilibration (intravenous)

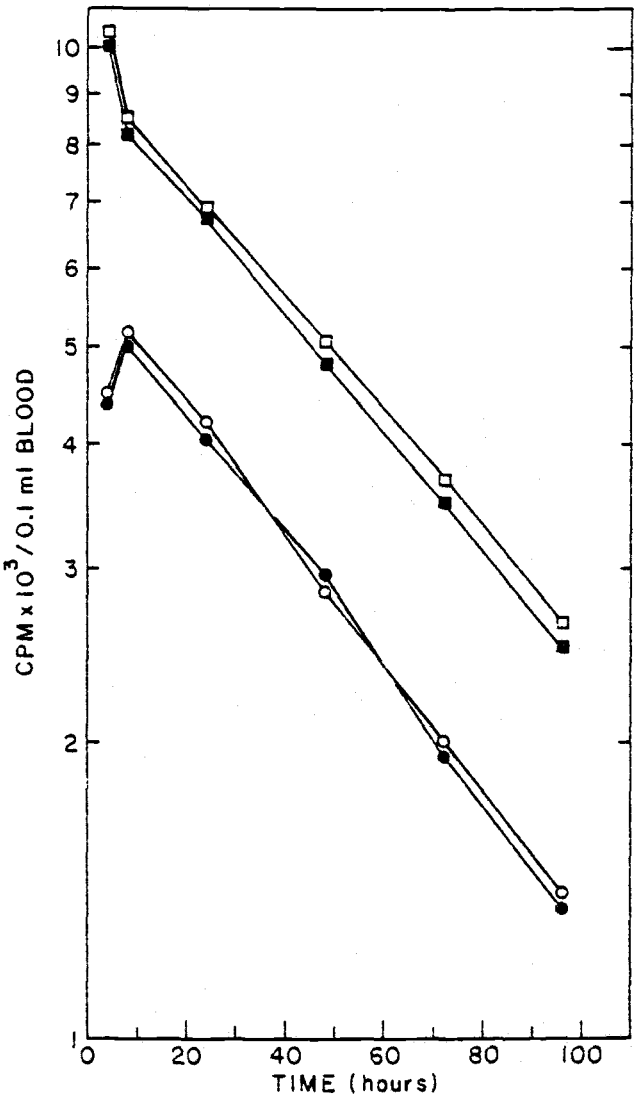


Figure 2. Blood absorption and elimination curves of ^{125}I-labeled purified tetrameric coho salmon immunoglobulin. Two fish received radiolabeled protein by intraperitoneal injection (o-o and ●-●), while two fish received an equivalent amount intravenously (□-□ and ■-■). All points represent an average cpm in duplicate 0.1 ml blood samples. Values obtained were normalized to a body weight of 100 g.

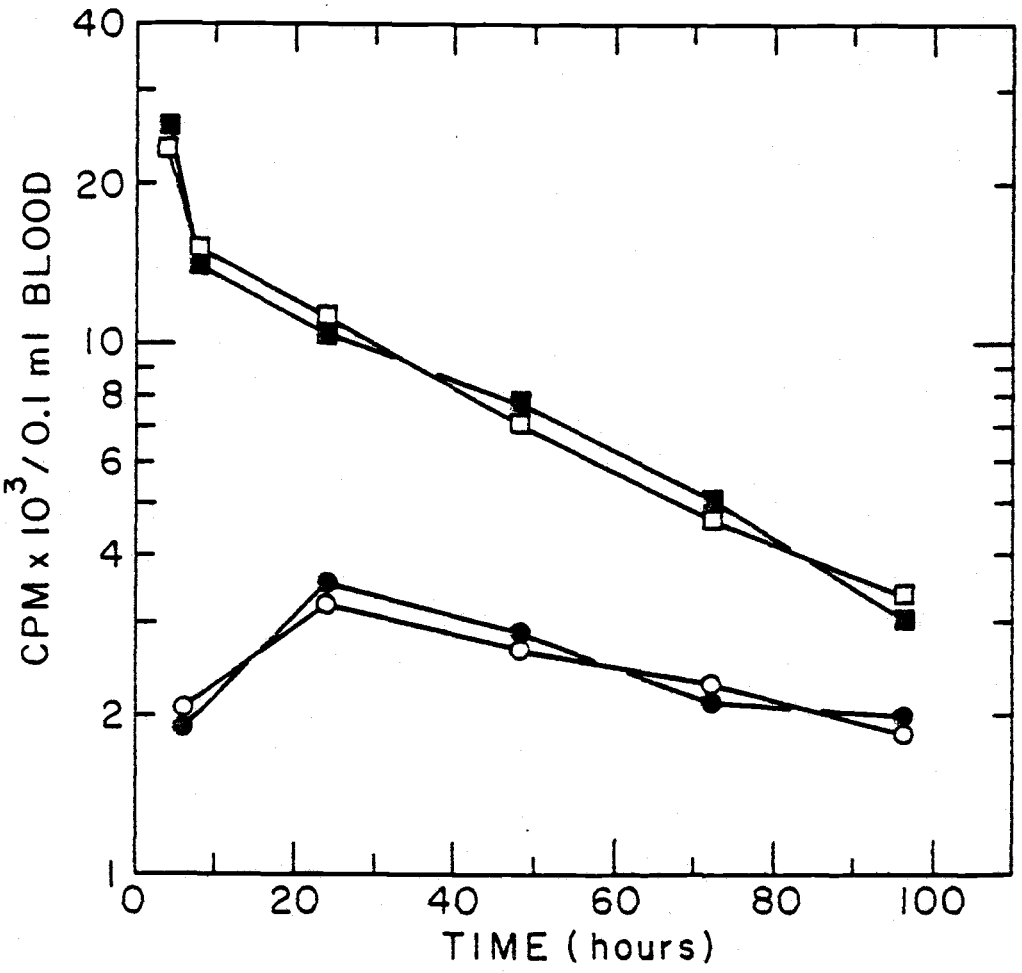


Figure 3. Blood absorption and elimination curves for ^{125}I-labeled monomer derived by reduction and alkylation of purified tetrameric coho salmon immunoglobulin. Two fish received radiolabeled monomer by intraperitoneal injection (o-o and •-•), while two fish received an equivalent amount intravenously (□-□ and ■-■). All points represent an average cpm in duplicate 0.1 ml blood samples. Values obtained were normalized to a body weight of 100 g.

and catabolic rates. The monomer also reached tissue equilibration by 8 h after intravenous injection while the absorbed material did not reach maximum levels until 24 h. At 8 h, the percent absorbed after intraperitoneal injection was 17% and at 24 h, 28% was absorbed relative to levels in intravenously injected fish. After 24 h, the monomer half-life following intraperitoneal injection averaged 96 h while the material given intravenously declined with a half-life of 44 h.

Protection of Fish Passively Immunized with Partially Purified Anti-*Vibrio anguillarum* Immunoglobulin

Coho salmon anti-*Vibrio anguillarum* immunoglobulin partially purified by chemical and chromatographic methods was effective for passively immunizing fish. The ammonium sulfate precipitated and molecular sieved preparation had an agglutination titer of 1:4 and 0.05 ml/fish was injected intraperitoneally in juvenile coho salmon (mean weight 2.8 g). Based on a total plasma volume of 5% of body weight (Randall, 1970), a four-fold dilution of the antibody was realized after absorption. The level of antibody in body fluids would have been below detectable limits by the agglutination assay, however a high level of immunity was conferred by this material. The data in Table 1 shows that 72% of the nonimmune fish died from vibriosis while only 11% from the passively immunized groups succumbed after water-borne challenge. This demonstrates that the partially

Table 1. Passive immunity to experimentally induced vibriosis in fish injected with partially purified antibody.

Treatment	Percent mortality caused by *V. anguillarum*[a]		
	Replicate 1	Replicate 2	Replicates combined
Immunized[b]	8	14	11
Nonimmunized	72	72	72

[a]Duplicate lots of 25 fish (mean weight 2.8 g/fish) from each treatment group were challenged by the water-borne method to 1.1×10^6 *V. anguillarum* cells/ml of aquarium water.

[b]Injected with 0.05 ml partially purified coho salmon anti-*V. anguillarum* immunoglobulin.

purified antibody was not significantly altered by purification treatments and that low concentrations of this immunoglobulin can provide high levels of protection.

Protection of Fish Administered Serum or Spleen Cells from Parenterally or Orally Immunized Donors

Passive immunity to vibriosis was demonstrated when serum or spleen cells were obtained from parenterally immunized donors while protection was not seen in fish receiving serum or cells from orally immunized or nonimmune donors. Table 2-A shows that actively immunized, bacterin-fed fish were protected (22% mortality) when compared to nonimmunized controls (52% mortality). Their protection was moderate in relation to parenterally immunized fish which had no mortality after challenge.

Data presented in Table 2-B shows that when each of these groups of fish served as serum or spleen cell donors for normal fish challenged with *V. anguillarum*, only serum from parenterally immunized fish (agglutination titer 1:1024) provided solid protection (0% mortality) and spleen cells from these same fish conferred a moderate level of immunity (20% mortality). Compared to the nonimmunized control fish (52% mortality), no significant statistical difference ($p \leq 0.05$) in mortality was observed in fish receiving materials from orally immunized or nonimmune donors. Recipients of serum from orally immune donors had 44% mortality and those receiving spleen cells had 42% mortality. Forty-six percent of the fish that had received serum from

Table 2. Efficacy of active and passive immunization against experimentally induced vibriosis in juvenile coho salmon.

A. Protection of fish actively immunized by oral and parenteral methods.

Method of immunization	Percent mortality caused by *V. anguillarum*[a]		
	Replicate 1	Replicate 2	Replicates combined
Parenteral[b]	0	0	0
Oral[c]	24	20	22
Nonimmunized	44	60	52

B. Protection of fish passively immunized with serum or spleen cells from actively immunized donors.

Method of donor immunization	Serum or cells transferred to recipients[d]	Percent mortality caused by *V. anguillarum*[a]			
		Replicate 1	Replicate 2	Replicates combined	p[e]
Parenteral[b]	Serum	0	0	0	0.01
Parenteral[b]	Spleen cells	20	20	20	0.01
Oral[c]	Serum	40	48	44	NS
Oral[c]	Spleen cells	44	40	42	NS
Nonimmunized	Serum	32	60	46	NS
Nonimmunized	Spleen cells	40	36	38	NS

[a]Duplicate lots of 25 fish from each treatment group were challenged by the water-borne method with 6.6×10^5 *V. anguillarum* cells/ml of aquarium water.

[b]Intraperitoneal injection with 2.6×10^8 killed *V. anguillarum* cells/fish. Mean weight at the start of active immunization was 14 g/fish for all groups.

[c]Received killed *V. anguillarum* bacterin in diet (1 mg bacterin/g of diet) for 15 consecutive days.

[d]Intraperitoneal injection with 0.5 ml donor whole serum or spleen cells.

[e]Probability level at which a significant difference is obtained relative to the nonimmunized group in Table A. Calculated values are less than, or equal to, the value given in the table. NS, not significant at $p \leq 0.05$.

nonimmune donors died and 38% of the recipients of spleen cells from these fish succumbed. Sera from the orally immunized and nonimmune donors had no detectable agglutination titer.

Immunity Induced by Oral or Water-borne Exposure of Fish to Bacterin

When an amount of bacterin equal to that incorporated into the daily feed ration of orally immunized fish was added to the aquarium water, essentially no protection was detected by challenge. Replicate 1 in Table 3 shows that orally immunized fish (16% mortality) had significant protection ($p \leq 0.01$) when compared to nonimmune controls (52% mortality) or to fish exposed to bacterin by the water-borne route (48% mortality). No statistical difference, at $p \leq 0.05$, was seen between nonimmune controls and those exposed by the water-borne route. Within replicate 2, however, the difference in mortality between nonexposed (72% mortality) and water-borne exposed (48% mortality) groups is significant at $p \leq 0.5$. The difference between the orally immunized (4% mortality) and both the control and water-borne groups is significant at $p \leq 0.01$. When statistical analyses were applied to the replicates combined, a significant difference was still discernable between the water-borne and nonexposed groups, but only at $p \leq 0.05$. For purposes of evaluating efficacy of immunization this difference would not be considered enough to recognize the fish exposed to bacterin by the water-borne method as protected. Agglutination titers to V. anguillarum were not detectable in the serum from any of the ten fish tested from each treatment group.

Table 3. Immunity induced by oral or water-borne exposure of fish to bacterin.

Method of exposure to bacterin	Percent mortality caused by *V. anguillarum*[a]			p[b]
	Replicate 1	Replicate 2	Replicates combined	
Oral[c]	16	4	11	0.01
Water-borne[d]	48	48	48	0.05
Nonexposed	52	72	62	-

[a]Duplicate lots of 25 juvenile coho salmon (mean weight 4.5 g/fish) from each treatment group were challenged by the water-borne method with 5.0×10^6 *V. anguillarum* cells/ml of aquarium water.

[b]Probability level at which a significant difference is obtained relative to the nonexposed group. Calculated values are less than, or equal to, the value given in the table.

[c]Fed 27 mg/day killed *V. anguillarum* cells in OMP diet for 15 consecutive days.

[d]Exposed to 27 mg/day killed *V. anguillarum* cells added to 68 l of aquarium water for 15 consecutive days.

Immunity Induced by Immersion of Selected Portions of the Fish Body in Bacterin

An immune response was elicited when the whole body, head or posterior body of juvenile coho salmon was immersed in either of two concentrations of bacterin (Table 4). All groups immersed in 4.0 mg/ml of bacterin were similarly protected (0-6% mortality) as were the fish that had the whole body or posterior body immersed in 0.4 mg/ml of bacterin (2% combined mortality). Fish that had the head immersed in 0.4 mg/ml of bacterin (16% mortality) had the highest mortality of all immunized groups. The mortality of every immersed test lot was significantly lower ($p \leq 0.01$) than in nonimmunized lots (64 and 52% mortality).

Serum agglutination titers against V. anguillarum were demonstrated in every immersed group. The 1:2702 mean titer in the nonanesthetized group that were entirely immersed in 4.0 mg/ml of bacterin was considerably higher than the 1:478 mean titer of similarly treated lots that were anesthetized before immersion. Antibody levels in the two groups that had the posterior body immersed in either of the bacterin suspensions were similar (1:32 and 1:34) and were comparable to those observed in fish that had the whole body immersed in bacterin at 0.4 mg/ml (1:32). The fish that had the head immersed had the lowest titers of all immunized groups, 1:23 and 1:16 at bacterin concentrations of 4.0 and 0.4 mg/ml, respectively. Titers against V. anguillarum were not detectable in nonimmunized controls.

Table 4. Immunity induced by immersion of selected portions of the fish body in bacterin.

Portion of body immersed	Bacterin conc (mg/ml)	Percent mortality caused by *V. anguillarum*[a]			Agglutination titer[b]
		Replicate 1	Replicate 2	Replicates combined	
Whole body[c]	4.0	4	0	2	2702
Whole body	4.0	0	0	0	478
Posterior	4.0	0	0	0	32
Head	4.0	0	12	6	23
Whole body	0.4	0	4	2	32
Posterior	0.4	4	0	2	34
Head	0.4	16	16	16	16
Nonimmunized	0	64	52	58	0

[a]Duplicate lots of 25 juvenile coho salmon from each treatment group (mean weight 10.7 g/fish) were challenged by the water-borne method with 2.0×10^5 *V. anguillarum* cells/ml of aquarium water.

[b]Reciprocal of the geometric mean of ten fish from each treatment.

[c]Not anesthetized prior to immersion while all other treatment groups were.

Exposure of Eggs to Bacterin During Artificial Spawning

Infection assays of young fish hatched from coho salmon eggs exposed to V. anguillarum during the process of artificial spawning revealed that recognition of the immunogenic material did not occur at this very early life stage or alternatively, it was not sequestered within the egg. Data in Table 5 shows that no statistical difference to V. anguillarum challenge was noted at $p \leq 0.05$ between the unexposed group and the test lots exposed to bacterin at any of the four fish weights tested. A trend which was noteworthy, however, was increased susceptibility to vibriosis in all three experimental groups as they increased in size up to 0.86 g.

Fish from each egg treatment lot demonstrated the capacity to recognize V. anguillarum immunogen later in life. The bacterin injected groups shown in Table 6 responded with solid protection (0% mortality) when compared to companion lots not receiving bacterin as juveniles (58-60% mortality).

Exposure of Embryonated Eggs to Bacterin by Injection or Immersion

An immune response was demonstrated in embryonated coho salmon eggs injected with V. anguillarum bacterin 4 to 5 days before hatching. No evidence of a response to the immunogen was detected in embryonated eggs immersed in bacterin at the same time. Serum agglutination titers of juveniles reared from these embryos (Table 7) showed that two of the six survivors from the injected embryo group had relatively high titers of 1:256 and 1:128 before receiving a second injection of the

Table 5. Susceptibility to experimentally induced vibriosis in coho salmon derived from eggs exposed to bacterin during artificial spawning.

Egg exposure to bacterin	Percent mortality caused by *V. anguillarum*[a]			
	Mean fish weight (g) at infection			
	0.67[b]	0.75[b]	0.86[b]	3.3[c]
Fertilized in bacterin	10	30	100	60
Water-hardened in bacterin	0	20	60	58
Unexposed	0	30	80	58

[a]At each size and in each treatment group fish were exposed to 2.0, 2.3, 4.0 and 1.0 x 10^7 *V. anguillarum* cells/ml of aquarium water by the water-borne method at 0.67, 0.75, 0.86 and 3.3 g, respectively.

[b]Ten fish from each treatment group were challenged.

[c]Duplicate lots of 20 fish from each treatment group were challenged.

Table 6. Test for immunological tolerance in fish derived from eggs exposed to bacterin during artificial spawning.

Egg exposure to bacterin	Percent mortality caused by *V. anguillarum*[a]	
	Bacterin injected[b]	Noninjected
Fertilized in bacterin	0	60
Water-hardened in bacterin	0	58
Unexposed	0	58

[a] Duplicate lots of 20 juvenile coho salmon (mean weight 3.3 g/fish) from each treatment group were challenged by the water-borne method with 1.0×10^7 *V. anguillarum* cells/ml of aquarium water.

[b] Intraperitoneally injected with 1.7×10^8 cells/fish of *V. anguillarum* bacterin 65 days after hatching.

Table 7. Serum agglutinin titers against Vibrio anguillarum in fish exposed to bacterin as embryos and by injection 218 days after hatching.

Embryo treatment[a]	Fish number	Agglutination titer[b] days post-injection of juvenile							
		0[c]	4	8	12	16	20	24	28
Unexposed	1	0	ND[d]	0	0	32	ND	8192	ND
	2	0	0	0	0	0	512	ND	4096
	3	0	0	0	0	0	512	1024	8192
	4	0	0	0	0	ND	ND	8192	8192
	5	0	0	0	0	0	64	8192	>8192
	6	0	0	0	0	16	512	1024	8192
	Geometric mean	0	0	0	0	3	304	3566	>7132
Immersed	1	0	ND	0	0	ND	256	2048	2048
	2	0	0	0	0	ND	1024	2048	8192
	3	0	0	0	0	0	128	512	8192
	4	0	0	0	0	8	ND	512	8192
	5	0	0	0	0	8	ND	1024	ND
	6	0	0	0	0	0	256	1024	8192
	7	0	0	0	0	0	512	4096	>8192
	Geometric mean	0	0	0	0	2	338	1248	>6474
Injected	1	0	0	0	0	0	512	>8192	>8192
	2	0	0	0	0	32	ND	ND	ND
	3	256	0	0	0	>8192	ND	ND	ND
	4	0	0	0	0	0	>8192	>8192	>8192
	5	0	0	0	0	16	8192	>8192	>8192
	6	128	0	0	512	8192	>8192	>8192	>8192
	Geometric mean	6	0	0	3	>57	>4096	>8192	>8192

[a]Unexposed, [b]immersed or injected with V. anguillarum bacterin as indicated 4-5 days prior to hatching; [b]Reciprocal of the titer; [c]Titer is a pre-injection value; [d]ND, indicates not determined.

bacterin on day 0 (218 days after hatching). All other fish from this and the other group, had no detectable agglutination titers.

After receiving an intraperitoneal injection of bacterin as juveniles 218 days following hatching, agglutination activity was lost for several days in serum of fish 3 and 6 from the embryo injected lot. A heightened response was then observed in these two fish as shown by 1:8192 titers on day 16 post-injection. At this time detectable titers were just appearing in other fish from the group and in those from immersed or unexposed embryos (0-1:32). After 20 days, three of the four other fish in the embryo injected group had titers of 1:8192 or greater while fish in the other lots still had titers ranging from 1:64 to 1:1024. By 28 days post-injection, titers of the majority of fish in all lots were approaching 1:8192. Six fish unexposed to bacterin as embryos and juveniles were sampled at the same 4-day intervals and had no agglutination titers throughout the duration of the test.

Effects of Temperature on Immunity and Infection

Growth Rate of *Vibrio anguillarum* at Selected Temperatures

Vibrio anguillarum grew in aerated BHI broth at 6, 12 and 18°C. Figure 4 is a plot of optical density versus incubation time at the three temperatures. Generation times based on optical density measurements at 520 nm in the logarithmic portion of the growth curves (Stanier, Doudoroff and Adelberg, 1970) were calculated to be 80, 151 and 442 min at 18, 12 and 6°C, respectively. These temperatures were chosen for certain of the temperature related experiments because 12°C is an estimated optimal temperature for juvenile Pacific salmon (Klontz, Brock and McNair, 1978) and 18 and 6°C represent extremes either side of the optimum that are not unduly stressful in laboratory studies.

Effect of Temperature on the Onset of Serum Agglutinin Formation

Production of agglutinating antibody in juvenile coho salmon immunized against *V. anguillarum* while held at three selected water temperatures was temperature dependent. Table 8 shows that agglutination titers were first observed on day 10 in fish held at the highest water temperature (18°C). At 12°C, agglutinins were initially detected in fish bled on day 15 while those held at 6°C showed no measureable levels of antibody until day 25. Once agglutinin formation had begun, the change in mean titers at each corresponding sampling interval was greater at each higher temperature.

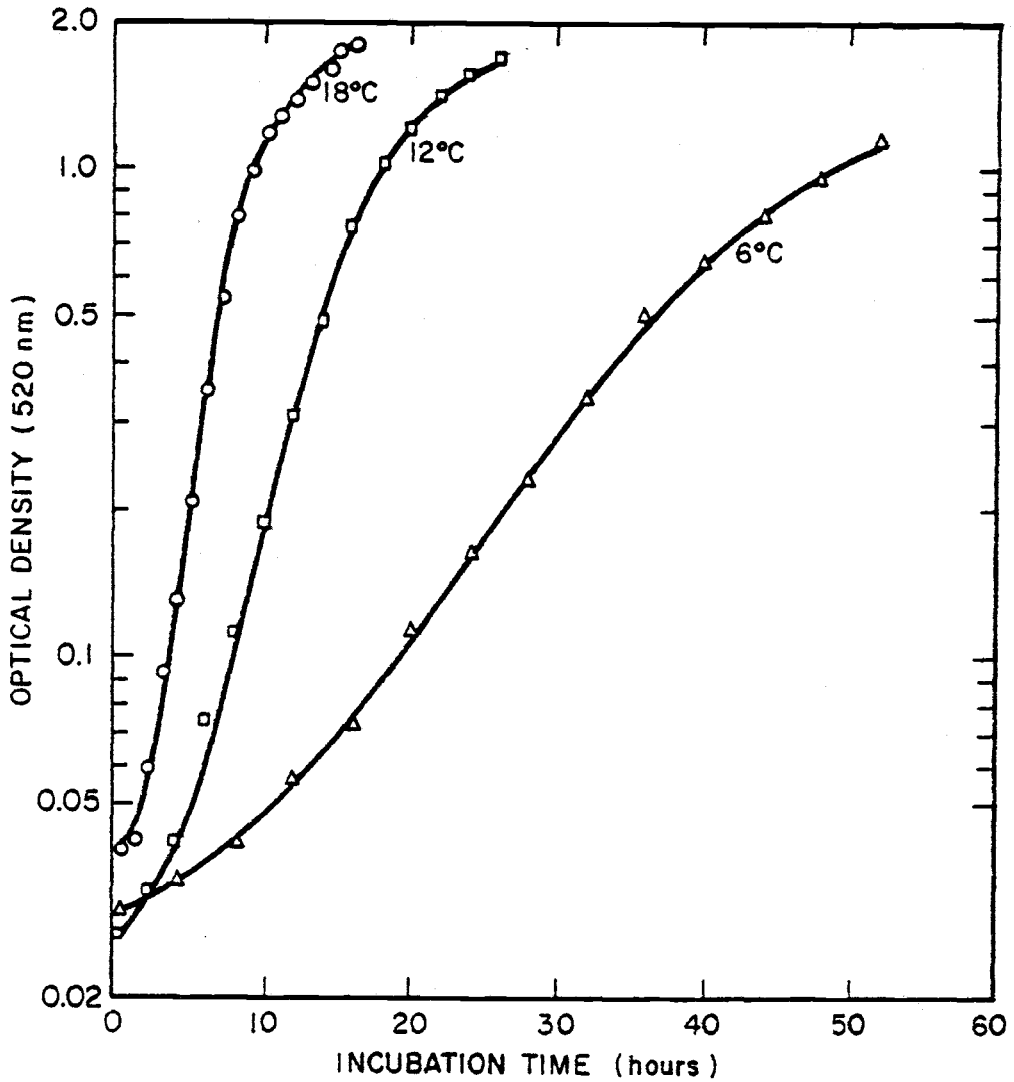


Figure 4. Growth curves of Vibrio anguillarum cultured in Brain Heart Infusion broth at 6 (Δ), 12 (□) and 18°C (o).

Table 8. *Vibrio anguillarum* agglutination titers in serum of fish following parenteral immunization[a] at three water temperatures.

Days post-immunization	Water temperature (°C)					
	6		12		18	
	Mean titer[b]	Range[c]	Mean titer[b]	Range[c]	Mean titer[b]	Range[c]
0	0	0	0	0	0	0
5	0	0	0	0	0	0
10	0	0	0	0	24	0-128[d]
15	0	0	56	16-128	147	32-512
20	0	0	169	64-512	588	512-1024
25	16	8-32	512	256-1024	2048	1024-8192
30	64	16-128	776	256-2048	3568	1024-8192

[a]Juvenile coho salmon (mean weight 11 g/fish) were intraperitoneally injected with 0.5 mg dry weight of *V. anguillarum* bacterin/fish.

[b]Reciprocal of the geometric mean titer of five fish.

[c]Reciprocal of the lowest and highest titer of five fish.

[d]One fish had a titer of 0.

Within a temperature group, the difference in mean titers was greater at succeeding sampling intervals and this affect was more pronounced at the higher temperatures. These observations suggest that the rate of increase in antibody production was greater at each higher temperature. A precise determination of the kinetics of this metabolic process in relation to temperature would require repeated sampling of individuals over a longer period of time.

Effect of Water Temperature on Mortality of Experimentally Infected Juvenile Coho Salmon

Groups of fish were experimentally infected with V. anguillarum while held at seven different water temperatures in 3°C increments (Table 9). The combined mortality of replicates decreased from 60% at 21°C to 4 and 0% at 6 and 3°C, respectively. A shorter mean time to death with increasing temperature was observed among groups at all temperatures at which mortality occurred.

Regression analysis of the mean time from exposure to death at each temperature revealed a linear relationship between water temperature and $\log_{10}$ of the mean time to death. The regression line so obtained was $Y = 1.4385 - 0.0497\ X$, where Y is the $\log_{10}$ of the mean time to death in days and X is water temperature in °C. The correlation coefficient of -0.8718 is signficant and an R^2 value of 0.7601 indicates that time to death was 76% temperature related.

Kidney smears from all surviving fish at each temperature were made on culture medium. Vibrio anguillarum was not recovered and bacterial colonies were observed from only one of 247 fish tested.

Table 9. Effect of water temperature on mortality of juvenile coho salmon experimentally infected with Vibrio anguillarum.

Water temp (°C)	Percent mortality caused by V. anguillarum[a]						Mean time from exposure to death (days)
	Replicate 1		Replicate 2		Replicates combined		
	Infected	Control	Infected	Control	Infected	Control	
21	56	0	64	0	60	0	2.7
18	60	0	56	0	58	0	3.7
15	28	0	52	0	40	0	4.6
12	24	0	32	0	28	0	6.1
9	8	0	24	0	16	0	11.8
6	4	0	4	0	4	0	23.0
3	0	0	0	0	0	0	ND[b]

[a]Duplicate lots of 25 fish (mean weight 19 g/fish) in each infected group were exposed to 2.0×10^6 V. anguillarum cells/ml of aquarium water at the indicated temperatures.

[b]ND, not determined because no fish died at this temperature.

Intraperitoneal Absorption Kinetics of Tetrameric Immunoglobulin at Selected Temperatures

The level and rate of absorption of purified tetrameric coho salmon immunoglobulin from the peritoneal cavity of fish was related to temperature. Comparisons between duplicates of animals administered the protein intraperitoneally or intravenously while held at each of three water temperatures (6, 12 and 18°C) are plotted in Figure 5. The ratio of the cpm in blood of intraperitoneally/intravenously (% absorption) injected fish at each temperature and sampling point is represented on the ordinate. All data were normalized to cpm/100 g body weight. At 18°C absorption was greatest and did not reach a maximum (78%) until 24 h after injection. After that time, the ratio of counts in the blood remained constant. Maximum absorption was complete by 8 h in fish held at 12 and 6°C as shown by the constant ratio of cpm after that interval. The amount of labeled protein absorbed at 12°C (59%) was greater than that absorbed at 6°C (37%).

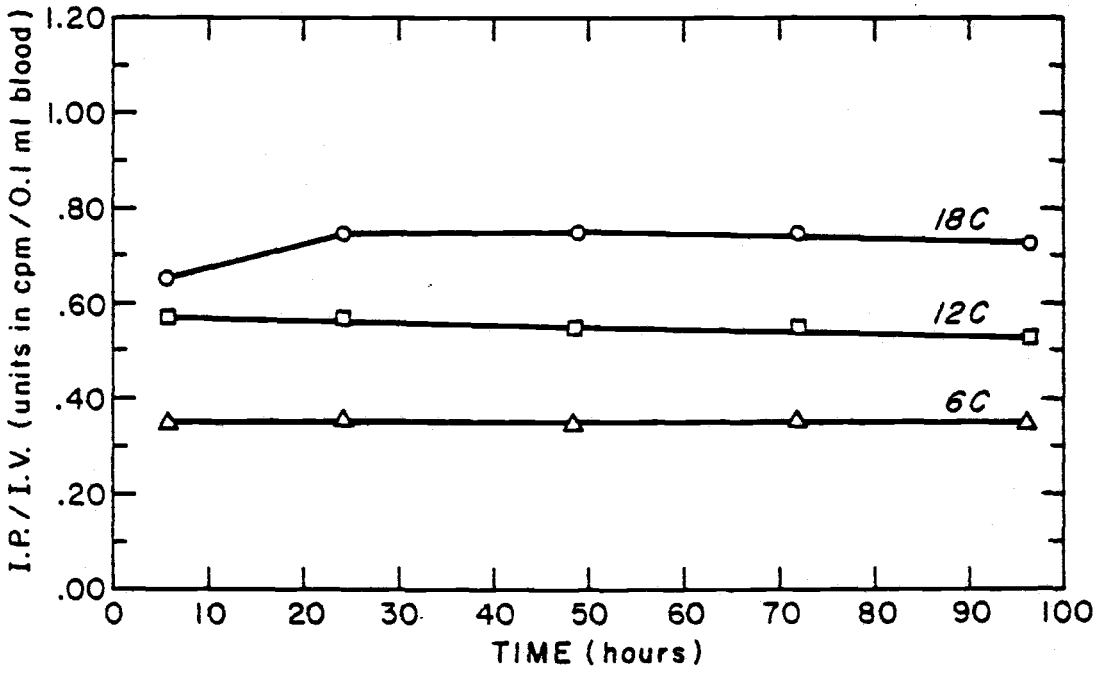


Figure 5. Plot of the ratio of ^{125}I cpm (radiolabeled tetrameric coho salmon immunoglobulin) per 0.1 ml of blood obtained from fish that received protein by intravenous or intraperitoneal injection while held at the same water temperature. Comparisons were made at three temperatures: 6 (Δ-Δ), 12 (□-□) and 18°C (o-o). All points represent a ratio of cpm intraperitoneal/ intravenous (ordinate) in duplicate 0.1 ml blood samples versus time (abscissa). Values obtained were normalized to a body weight of 100 g.

Serological and Immunological Methods

Purification of Juvenile Coho Salmon Serum Immunoglobulin

Purified juvenile coho salmon serum immunoglobulin was obtained by affinity and molecular sieve chromatography using the method O'Leary (1980) developed for purifying rainbow trout immunoglobulins. Purity of the coho immunoglobulin was confirmed by SDS-polyacrylamide gel electrophoresis and results of this assay are shown in Figure 6. The purified antibody preparation was run on the right and molecular weight markers on the left. In descending order of molecular weight, the markers are: 92,500 (phosphorylase B), 66,200 (bovine serum albumin), 45,000 (ovalbumin), 31,000 (carbonic anhydrase) and 21,000 (soybean trypsin inhibitor) daltons. Only two bands are seen in the antibody sample with estimated molecular weights, relative to standards, of 76,000 (H) and 29,000 (L) daltons. These bands are the dissociated heavy and light chains of the antibody molecule (Cisar and Fryer, 1974). Purity of the antibody preparation was also demonstrated by a single precipitin band obtained when the purified coho immunoglobulin (Ig) was electrophoresed and allowed to react with rabbit antiserum specific for coho whole serum (Anti-Co Serum) (Figure 7). In contrast, multiple precipitin bands are seen in the reaction between Anti-Co Serum and ammonium sulfate precipitated coho serum (AS).

Figure 6. SDS-polyacrylamide (10%) gel electrophoresis of purified coho salmon serum anti-TNP immunoglobulin (right) and molecular weight markers (left). Electrophoresis was run at 6 ma for 19 h. Molecular weight markers are: A) phosphorylase B (92,500 daltons), B) bovine serum albumin (66,200 daltons), C) ovalbumin (45,000 daltons), D) carbonic anhydrase (31,000 daltons) and E) soybean trypsin inhibitor (21,000 daltons). Heavy and light chains of coho salmon serum immunoglobulin are H (∿76,000 daltons) and L (∿29,000 daltons), respectively.

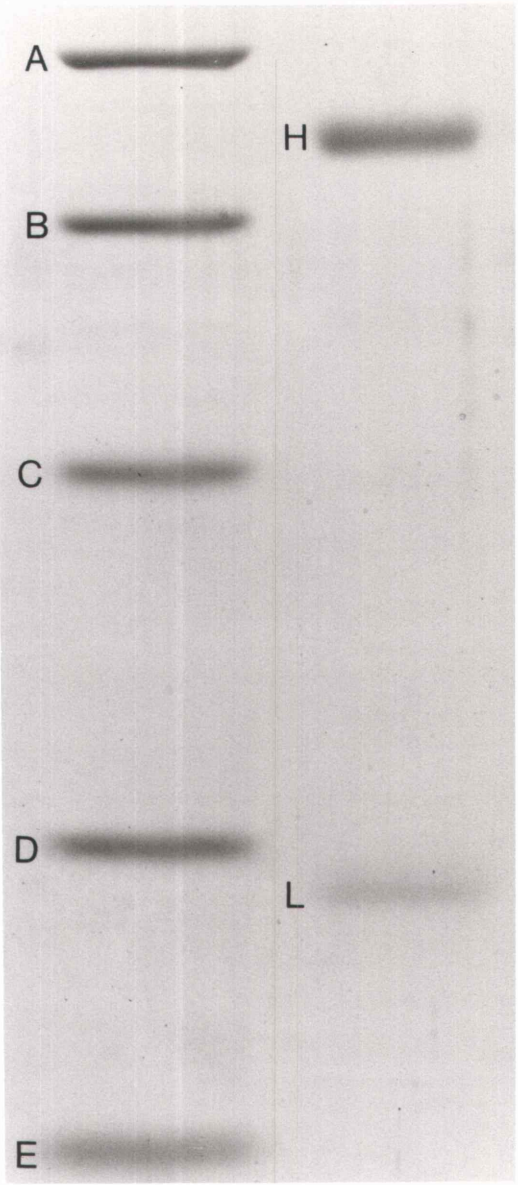


Figure 6

Figure 7. Immunoelectrophoresis results of purified juvenile coho salmon serum anti-TNP immunoglobulin (Ig) and ammonium sulfate precipitated coho serum (AS) diffused against rabbit antiserum specific for coho salmon whole serum (Anti-Co Serum). Anode to the right.

Figure 8. Immunoelectrophoresis results of juvenile coho salmon whole serum (Co) and body mucus concentrate (M) diffused against rabbit antiserum specific for coho salmon serum immunoglobulin (Anti-Co Ig). Anode to the right.

Figure 7

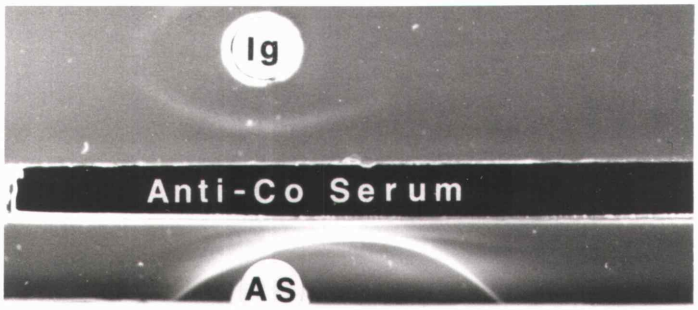
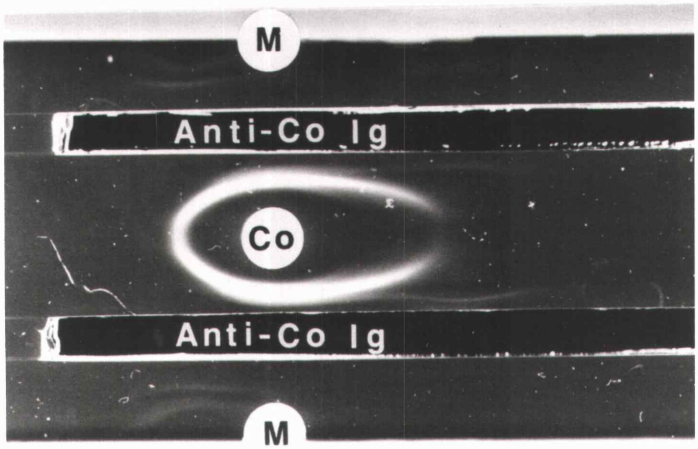


Figure 8



Production of Rabbit Antiserum Specific for Coho Salmon Serum Immunoglobulin

Purity of the rabbit antiserum made against coho salmon serum immunoglobulin specific for the TNP hapten is shown by the immunoelectrophoretic pattern in Figure 8. The intense band around the center well inoculated with coho whole serum (Co) represents the specific reaction of coho immunoglobulin with rabbit antiserum specific for the coho serum immunoglobulin (Anti-Co Ig) that was placed in the troughs.

Immunoelectrophoretic Assay for Immunoglobulin in Fish Mucus

Two components that reacted with rabbit antiserum specific for the coho serum immunoglobulin (Figure 8) were observed in electrophoresed mucus concentrates (M). These entities appear to have similar electrophoretic mobilities and one diffuses at a faster rate than the other. The lower intensity of the mucus derived bands compared to the serum precipitin bands suggests the components in mucus were less concentrated or had significant antigenic heterogeneity to the rabbit antiserum.

DISCUSSION

Coho salmon (O. kisutch) and V. anguillarum comprise an excellent host-pathogen system to study processes of infection and immunity. Coho salmon are naturally infected with V. anguillarum in the marine environment (Harrell et al., 1976; Sawyer and Strout, 1977; Tajima et al., 1981) and the reproducible water-borne exposure method simulates the infectious process as it occurs in nature (Ransom, 1978). The juvenile coho salmon is an ideal salmonid for experimental applications because it adapts well to laboratory conditions and non-specific mortality during long holding periods is minimal. Predictable immunological responses can be achieved with coho salmon because previous investigators (Spence et al., 1965; Cisar and Fryer, 1974; Paterson and Fryer, 1974a, 1974b; Rohovec, 1974; Gould, 1977; Udey, 1978) have characterized many of these responses using antigens of V. anguillarum and Aeromonas salmonicida, two important Gram-negative fish pathogens. Juvenile coho salmon reared in freshwaters of the Pacific Northwest can be presumed immunologically naive to V. anguillarum because the bacterium is known only to inhabit marine environments in this region. Protection trials and immunological assays, therefore, are not complicated by latent immunological effects in the host resulting from previous contact with the bacterium. The antigens of V. anguillarum elicit immune responses that are generally conspicuous and results are usually unequivocal. These studies were designed to expand upon knowledge relating to fish immunization by comparative methodology, to further describe characteristics of the coho salmon

immune system with antigenic material from an important fish pathogen and to examine certain parameters of infection and immunity as they relate to environmental temperature.

Passive immunization, while not a practical technique for most fish cultural activities, was an effective in vivo method for the study of the coho salmon immune system. The catabolic rate of intravenously administered coho salmon tetramer has been shown to be relatively rapid with a half-life of about 40 h at 12°C (Voss et al., 1980). A rapid catabolic rate is also characteristic of IgM in higher vertebrates, albeit mostly determined in homeotherms. Advantage was taken of the catabolic rate data to study the membrane transport kinetics of tetrameric immunoglobulin from the peritoneal cavity into the serum. The purpose was to quantitate the amount of antibody absorbed so related passive immunization experiments could be evaluated. Moreover, further information on the dynamics of the coho immune system would be available for comparative purposes. The tetramer was efficiently absorbed within 8 h and effective distribution of this material throughout the vascular system after intraperitoneal injection could be expected. The kinetics of absorption were shown to be clearly temperature related and with these data it is possible to anticipate how much antibody might be absorbed at different temperatures. Efficacious absorption of a tetrameric antibody in fish is curious because in milk-suckling mammals, maternally derived monomeric IgG is selectively absorbed from the gut (Jones and Waldman, 1972). This could imply that analogous monomeric species are not present in coho salmon.

A monomeric immunoglobulin has not been described in salmonids although the possible existence of such an entity in serum or body secretions has not been unequivocally ruled out. To address this possibility, the kinetics of absorption of monomeric subunits obtained from the tetramer were examined. Inferences concerning the probable presence of a monomeric antibody could be made using this indirect approach. The monomer was absorbed ineffectively, and indeed, the long half-life of the protein in intraperitoneally injected fish indicated that absorption continued over the entire 96 h sampling period. This observation suggests that a monomer, having extensive structural homology to subunits of the tetramer, might not be expected in body fluids since transport of such a protein would be inefficient. An efficiently transported tetramer may be more plausible and, in fact, a tetrameric immunoglobulin has been reported in mucus of the Florida gar, Lepisasteus platyrhineus (Bradshaw, 1971).

It was necessary to demonstrate that the coho salmon tetrameric immunoglobulin could still confer protection after purification. Possible chemical modifications or conformational alterations during its preparation might have affected its catabolic rate and absorption kinetics or both. Results indicated the immunoglobulin was not significantly altered by purification methods since it conferred high levels of immunity to passively immunized fish. This antibody was not iodinated while the protein for the absorption studies was, however, limited iodination is recognized as a minimal chemical modification to immunoglobulins and has been routinely used to label such proteins for metabolic studies (Williams and Chase, 1967).

A rather striking result from this experiment was the low titer of specific V. anguillarum antibody that afforded a high level of protection by adoptive transfer. Presuming 60% of the material was absorbed and a 1:4 dilution after its equilibration in the vascular system, the antibody in undiluted serum of injected fish would have been undetectable by macroscopic agglutination methods. Even at this low level, however, immunity was apparent and not unlike protection seen in certain actively immunized fish (Gould et al., 1978; Egidius and Anderson, 1979; Itami and Kusuda, 1980). It might be feasible to attempt passive immunization with sera or extracts from body secretions, as an in vivo assay for suspected nonagglutinating or secretory immunoglobulins.

The presence of nonagglutinating serum components in orally immunized salmonids was considered. If indeed, a serum entity was an element of the orally induced immune response, it could be measured in vivo by passive immunization with orally immunized fish serving as serum donors. Further, if a generalized cell mediated response resulted from oral immunization, it too might be determined by the same method using spleen cells from immunized donors. No evidence of protection was observed; however, in fish receiving serum or spleen cells from their orally immunized counterparts. The possibility remains that an inducible substance was present in the serum at very low levels and an amount sufficient to afford protection was not transferred. Lobb and Clem (1981) have, in fact, reported the existence of two populations of immunoglobulin in the sera of the

sheepshead (Archosargus probatocephalus), a marine teleost. The smaller antibody was a 6S monomer present at 1/20th the concentration of the predominant tetrameric 16S molecule. Moreover, given the slow rate of absorption of the monomer obtained by reduction of the tetramer, a naturally produced serum monomer may have been absorbed too slowly to protect fish challenged soon after receiving the serum. Generally, though, the results indicate the lack of a generalized whole body response as a result of oral immunization. The earlier observation of Fletcher and White (1973) suggesting a mechanism of gut-associated immunity in response to oral immunization of plaice, appears relevant to the salmonid system.

Transfer of immunity with serum and spleen cells from parenterally immunized donors was meant to serve as a positive control segment of the experiment. At the time of these studies, however, adoptive transfer of immunity with spleen cells in salmonids had not been documented. Spence et al. (1965) had clearly demonstrated the capability to transfer immunity against furunculosis between salmonids with hyperimmune serum. In a recent study, immunity to vibriosis by adoptive transfer was shown in rainbow trout that received plasma, pronephros or spleen cells from the same species which had been immunized by immersion (Viele et al., 1980). Plasma provided the highest level of protection, similar to serum-conferred passive immunity reported here, with pronephros and spleen cells rendering less, although significant, protection. With the experiments described here and this recent report, passive immunization data are available

with donor fish immunized against vibriosis by oral, parenteral and immersion techniques.

During the course of the oral immunization studies, reports began to appear that documented a strong generalized immune response in salmonid fish hyperosmotically infiltrated (Croy and Amend, 1977), sprayed (Gould et al., 1978) or immersed (Gould et al., 1979) with V. anguillarum bacterins. The common feature between these methods was that the external body surface of the fish was exposed to bacterin. Realizing the apparent immunological potency of V. anguillarum antigens and the effective response realized by these new methods, it became important to demonstrate that the response from oral immunization was not simply a subtle manifestation of immunity induced by exposing the fish body surface to bacterin. Conceivably, orally administered bacterin could leach into the aquarium water from the diet or fecal material during the 15 day feeding regimen and produce a low level of prolonged exposure of the external body surface to antigen. A modest protective response similar to that produced with oral immunization might be expected under these circumstances. The results of the experiment to test this hypothesis indicate that orally induced immunity is indeed different than an immersion induced response. The significant differences in mortality between replicates of orally immunized and fish exposed to bacterin by water-borne exposure confirm that leaching of bacterin from diet or feces could not account for orally induced immunity. Even while assuming leaching of the entire portion of bacterin from the diet, protection of fish exposed to

water-borne bacterin was essentially nonexistant. The orally immunized lots, however, had high levels of protection compared to either nonimmunized, or water-exposed groups, that were also seemingly nonimmunized.

Having determined that a generalized immune response in orally immunized fish was not apparent by the passive immunization experiments, and that an orally induced response was not quasi-immersion, it is possible to postulate a mechanism for oral immunity. Recalling that lymphoid tissues and cells occur in the gastrointestinal tract of all vertebrates (Cohen, 1975) and that Fletcher and White (1973) described gut-associated antibody in plaice, an intestinally associated mechanism seems likely.

Such tissues and cells are effectors of localized immunity with cellular and secretory components. It seems plausible that these immune functions evolved, and are extremely important, in fish because they are in intimate contact with the medium that may also contain infectious agents. The first line of defense to infectious organisms in all animals is the external and internal body surface and with fish it is reasonable to surmise that specific immune mechanisms evolved there. Further, Ezeasor and Stokoe (1980) described structures in the gut of rainbow trout as cast in the mode of a lymphoid organ that developed in the course of feeding and growth in young fish. This development could be in response to ingested antigenic material and to the microbial flora that becomes established with the onset of feeding. Oral administration of bacterin could

presumably stimulate such gut-associated lymphoid tissues and provide localized protection to pathogens that enter the host through the gut.

The magnitude of the response produced by immersion indicated that if localized immunologically functional sites could be identified, in vitro assays with cells or secretory substances from these areas might be informative. Further, immersing selected portions of the fish body in bacterin could shed light on the route for uptake of antigen and for identifying tissues important for localized immune responses. Several observations can be made from the results of the experiments concerning immersion of selected portions of the fish body in bacterin. A generalized response, evidenced by humoral agglutination titers, was seen in all four groups that had their head or posterior body immersed. Fish with agglutination titers of 1:32 were solidly protected and as these titer values decreased to 1:16, protection was nearly as high, but beginning to wane. In general, immunity in the groups that had just their heads immersed was less than in the other immunized groups at the same bacterin concentration. Comparisons between fish entirely or only partially submerged reveal that at the higher bacterin concentration, the agglutination titer was much higher in the fish completely immersed. In the diluted bacterin the differences in mean titers between all groups were small. The reason for this disparity has to do with the kinetics of immunological responses at different antigen concentrations. A description of these kinetics is relatively speculative in mammals (Nossal and Ada, 1971) and would certainly be so in this case. The data does suggest, however, that the optimal

antigen dosage for induction of serum agglutinin formation by this immersion technique is nearer to the higher bacterin concentration, 4.0 mg/ml. A final observation was, while protection of anesthetized and nonanesthetized lots entirely immersed were similar, the higher mean titers in the nonanesthetized fish suggests more efficient exposure to bacterin in this group. Support for this hypothesis has been described by Anderson et al. (1979) using rainbow trout immersed in a suspension of the O-antigen extracted from the bacterial pathogen, Yersinia ruckeri. They observed fewer O-antigen specific plaque forming lymphocytes from spleens of trout anesthetized prior to immunization. Inhibition of lymphatic circulation as a result of the effect of the anesthetic on a neuro-muscular lymphatic pump in the tail (Kampmeier, 1969) might account for these observations.

The lateral line was the organ where bovine serum albumin was most concentrated in rainbow trout hyperosmotically infiltrated with this soluble protein (Amend and Fender, 1976). The gills were secondary in terms of the quantity of albumin detected and only trace amounts of the substance were found in the stomach and intestine. The authors postulated that the protein was sequestered in the lateral line, functionally a sensory organ, and absorbed for about 6 h from that site into a lymph duct directly beneath it. Bowers and Alexander (1981) showed that a particulate substance, Escherichia coli whole cells, was found in the gills of brown trout (Salmo trutta) infiltrated by methods similar to those advanced by Amend and Fender (1976). They found no evidence of the bacterium in the lateral line. In neither study,

however, was a correlation established between presence of the test material in various tissues and the nature of an immune response in the animal.

The V. anguillarum bacterin used in the studies reported here, consists of both particulate and soluble bacterial components. Although the major portion of the lateral line was excluded from exposure in the groups that had their heads immersed, nares and lateral line extensions are located in the head (Lagler et al., 1962) and may have served as deposition sites for soluble bacterin components. If particulate antigens are taken up exclusively across the gill tissue by immersion immunization, it is possible to speculate that soluble V. anguillarum antigens are more important than particulate antigens for inducing protection, because generally lower levels of immunity were observed in fish having only their head immersed. A direct approach to verify the nature of the protective antigens would be to compare immunity between groups of fish exposed to separate soluble and particulate bacterin components. Possibly, if recognition of soluble antigens is important for protective immunity, oral immunity is not as effective because the soluble antigens are substantially inactivated by the low pH of the fish stomach and proteolytic gastrointestinal enzymes.

Occurrences of certain viral and bacterial diseases are typically associated with very young salmonids (Pilcher and Fryer, 1980; Wood, 1974). A body of circumstantial evidence exists suggesting that the infectious agent associated with many of these diseases resides in or on the egg, or possibly sperm cells, and is vertically transmitted from the male or female parent to its progeny. If infectious organisms can

persist in association with the egg and developing embryo, it was conjectured that V. anguillarum antigens might also endure if they could be sequestered within the egg during spawning. Retained antigens might then be recognized as foreign or self as immunological competency was attained, and an immune response or lack of it later in life might reflect upon the nature of an earlier immunological event. Immersion of eggs in bacterin at spawning offered a means to test these possibilities. Results of the experiment with eggs exposed to bacterin during artificial spawning indicated that recognition of the antigens did not occur. Several possibilities could explain the lack of definitive results. The bacterin may not have become egg-associated and was simply washed away. Possibly there was not enough of it to persist and induce a response later. Metabolic activities of the egg during maturation may have degraded the antigen so that it was not present when immunological competency was attained. Whatever the reason, both the lack of enhanced protection and no apparent tolerance to the antigenic material suggest that recognition of V. anguillarum antigens was not realized.

An interesting trend was noted, however, as a result of infecting the young fish at different sizes. Susceptibility to vibriosis appeared to increase as the fish grew from a mean weight of 0.67 g to 0.86 g. Although the concentration of the exposure dose also increased slightly, this variation was not considered enough to account for the increased mortality pattern. In fact, the fish infected at 1.2 g were exposed to half as many organisms as those infected at 0.67 g, and yet mortality was significantly higher at the larger size.

The possibility for egg-derived material to provide enhanced resistance of alevins to infectious agents has recently been reported by Yoshimizu et al. (1980). They showed that the yolk of alevin and sac fry was sterile as determined by the lack of aerobic microflora. At 0.67 and 0.75 g the alevins in these studies were what are commonly referred to as yolk-sac fry; that is, fish carrying most of the yolk material before it was absorbed. At 0.86 g this material was no longer observable and these coho were essentially similar in their susceptibility to vibriosis as when they were larger. It appears, therefore, that egg-derived material or certain physiological factors were responsible for the resistance in these alevins. If very young salmonids are immunologically competent and yet refactory to infection by many of their pathogens, immunizing them with moderately attenuated preparations may be feasible.

While exposure of eggs to bacterin at spawning to induce protection in the resulting juveniles may have proved impractical, exposure of developing embryos to immunogenic material seemed to have merit. Two viral diseases of Pacific salmon, infectious pancreatic necrosis and infectious hematopoietic necrosis, most frequently cause devastating losses in very young fish. The concept of immersion offers a possible means whereby the delicate embryos, through immersion of the egg, could be exposed to antigens from these agents. Inducible protective responses would thus have been provoked as the alevins emerge at hatching. Lymphoid tissues and cells have been described in Atlantic salmon (Ellis, 1977) and rainbow trout embryos (Grace and Manning, 1980), therefore, induction of an immune response late in embryonic development

was plausible. The potent antigens of V. anguillarum provided a reliable system to test these possibilities in coho salmon embryos. Although none of the fish reared from embryonated eggs immersed in bacterin 4-5 days before hatching showed evidence of immunological provocation, two of six from the injected egg group clearly showed residual effects indicative of a previous encounter with V. anguillarum antigens. These two fish had serum agglutinin titers 218 days after hatching and their only opportunity to contact V. anguillarum antigen was when the yolk material of the embryo was injected before hatching. Late in incubation, the yolk material of a salmonid egg has a vascular network which is derived from the embryo. The yolk serves as a source of sustenance for the animal until feeding begins (Hoar and Randall, 1969). Potentially, the two fish having residual titers to V. anguillarum represent only those in which the vascular network of the yolk was penetrated by injection. Alternatively, ontological development may have been such that only these embryos were immunologically competent. The immersed embyros and nonresponsive injected embryos apparently lacked a primary response as embryos, and a secondary or tolerance response as juveniles, therefore either alternative is possible. The eggs were collected at the same time, reared under identical conditions and hatched within a 2 day period. Developmentally, then, they were very similar at the time of exposure to bacterin and the lack of appropriate exposure to bacterin seems more likely. Modifications to the basic immersion method, therefore, might be explored to encourage penetration of immunogenic material

into the embryo vascular system. Prolonged exposure, hyperosmotic or vacuum infiltration and membrane solvents are some adaptations to the basic method that warrant consideration. Additionally, soluble protein antigens might be more effectively administered with modifications of the basic immersion method.

Two additional observations are noteworthy concerning the fish reared from the responsive embryos. After receiving an intraperitoneal injection 218 post-hatching, all evidence of agglutinating antibody disappeared for 12-16 days, however by day 16, both fish had titers of 1:8192 or greater. The loss of agglutination activity can be explained by postulating the formation of antigen-antibody complexes consisting of particulate or soluble V. anguillarum antigens and specific humoral antibody that became localized in lymphoid tissues (Nossal and Ada, 1971). Agglutination activity reappeared as the antigen was metabolized and antibody became excess to that bound by the antigen. One wonders if the disappearance of humoral antibody coincides with loss of protection for some period of time and what then might happen if the pathogen were present. This possibility should be further examined and considered when mass immunization of fish is conducted. The rapid rise in titers to high levels (1:8192) is characteristic of a secondary immune response in an animal that has previously encountered an antigen. This further substantiates that these fish had previous contact with the antigen.

The discussion has focused upon infectious processes as they relate to immunity in coho salmon, and certainly, immunity profoundly

affects the nature of infectious processes in any host-pathogen system. The outcome of such an interaction, however, depends on many properties of the pathogen, numerous host-determined factors, and the environment. Excellent interpretations of this concept as applied to infectious diseases of fish have been presented by Snieszko (1974, 1978). An important component of the environment for ectothermic vertebrates is temperature.

The growth kinetics of V. anguillarum in relation to temperature suggest that it is best classified a psychrotrophic bacterium by the criteria of Morita (1975). The organism is capable of reasonably rapid growth at low temperatures, evidenced by the growth curve obtained at 6°C, while its optimal growth temperature is reported to be above 15-20°C (Hendrie, Hodgkiss and Shewan, 1971). In vitro growth of the organism within a temperature range commonly encountered by juvenile salmonids was clearly shown in these studies. Undoubtedly, the growth dynamics of the organism within a host are affected by a myriad of factors and the in vitro growth data is only suggestive of what may occur in vivo. The capacity of the bacterium to undergo relatively rapid growth at low temperatures, however, infers that lethal infections can develop under these conditions, particularly if host immunity is depressed.

The potential for repression of immune function at reduced temperatures is known (Avtalion, 1973) and with the knowledge that the pathogen could proliferate at lowered temperatures, it was pertinent to evaluate the appearance of pathogen-specific antibody in immunized

fish held at selected temperatures. Although serum agglutination titers depend upon and arise after cell-mediated events have occurred, titers do reflect, both temporally and quantitatively, the impact of temperature on immune function. The results presented here revealed that as water temperature was decreased, the primary response, measured by the appearance of serum agglutinins, was distinctly delayed. Similar data was previously described for juvenile coho salmon with bacterins produced from the agents of furunculosis, A. salmonicida (Paterson and Fryer, 1974b; Fryer et al., 1976); and bacterial kidney disease, Renibacterium salmoninarum (Fryer et al., 1976). The magnitude and rapidity with which agglutinin levels increased were clearly temperature related. In those studies, though, background agglutination titers to both organisms were apparent in nonimmunized control animals. This suggests that a primary response was not being measured since the fish presumably had previous or ongoing contact with antigens from these bacteria. In the experiments for which A. salmonicida endotoxin was the immunizing antigen (Paterson and Fryer, 1974b), however, the kinetics of agglutinin formation were markedly similar to those described here with V. anguillarum bacterin. This indicates, on the other hand, that a primary response was, indeed, being observed and that the background agglutinins were naturally occurring serum constituents. The bacterins used in the 1976 report (Fryer et al.) were given as emulsions in Freund's Complete Adjuvant and initial bleedings were performed 15 days after immunization. It is not clear, therefore, whether or not loss of detectable serum

agglutination activity occurred in a manner described here when juveniles held at 12°C and having V. anguillarum titers were again immunized. Administering the bacterins in adjuvant affords low-level, prolonged release of the emulsified material and thus possibly sufficient antigen was not available to bind all presynthesized antibody. The phenomenon of the disappearance of pre-existing agglutinins after injection of antigen was shown by Paterson and Fryer (1974b) with fish held at 6.7°C but not in those maintained at 12.2 or 17.8°C. The dialogue that has proliferated from attempts to immunize fish is somewhat confusing because reports of success and failure reveal some inconsistencies. These and other studies indicate that the added complexity of environmental temperature make interpretations of host-pathogen interactions in ectotherms even more difficult than in their homeothermic counterparts. Perhaps, relationships between the host, pathogen and environment need to be more critically evaluated when fish are immunized against ubiquitous pathogens.

Septicemic infections caused by Gram-negative bacteria in salmonid fish increase in severity at elevated water temperatures. Experimentally, a shorter mean time to death and higher mortality were observed at increased water temperatures in several species of Pacific salmon infected by A. salmonicida and A. hydrophila (Groberg et al., 1978). The results described here are very similar to those reported with juvenile coho infected by A. salmonicida. Vibrio anguillarum also produces a hemorrhagic septicemia in coho salmon (Harbell et al., 1979)

and its growth dynamics compare to those of A. salmonicida, a psychrotroph, that similarly produced fatal infections at lowered temperatures (Groberg et al., 1978). Although vibriosis epizootics have generally occurred at elevated water temperatures, the organism can potentially survive, grow and cause disease in salmonids at temperatures below 12°C, particularly if immunological responses of the host are delayed.

Throughout these studies efforts were made to conclusively demonstrate the occurrence of immunoglobulin in the external body mucus of juvenile coho salmon. Inactivation of pathogens entrapped in mucus would likely be more desirable than when a disease-causing organism had begun to proliferate within tissues. Higher vertebrates possess a variety of antibodies in secretions (Tomasi, 1976) and reports of secretory immunoglobulins in other fish species encouraged these efforts (Fletcher and Grant, 1969; DiConza and Halliday, 1971; Bradshaw et al., 1971; Fletcher and White, 1973; Hines and Spira, 1974; Ourth, 1980). The secretory antibodies in these earlier studies were either not characterized or determined to have been very similar to their serum analog. Cautious interpretation of these results was necessary because the secretory protein may have simply represented a transudate of the serum immunoglobulin and mucus collection methods could have significantly affected this phenomenon. A preliminary report has only recently been made documenting the occurrence of immunoglobulins in the bile and mucus of the sheepshead (A. probatocephalus), a marine teleost, that are distinctly different than its two serum immunoglobulins (Lobb, 1980).

Purified coho salmon tetrameric serum immunoglobulin specific for a single haptenic molecule was obtained so that rabbit anti-coho immunoglobulin serum could be prepared and used to probe mucus secretions for cross-reactive components. Immunoelectrophoretic methods were used on the assumption that sufficient homology would exist in the domains of different immunoglobulin populations of the coho so that cross-reactions could be expected. Support for this is provided by the work of Lobb (1980) cited previously. A further assumption, was that the purified serum immunoglobulin was a homogeneous, tetramic class of immunoglobulin. The electrophoretic behavior of the protein in a reducing gel revealed only two bands corresponding to heavy and light immunoglobulin chains, and a single precipitin band was discernable when it reacted with rabbit antiserum specific for coho whole serum. These data are indicative of purity. Detection of subpopulations within the same immunoglobulin class, however, may not have been achieved by these methods. Lobb and Clem (1981) could only demonstrate the two HMWIg subclasses in the sheepshead by analytical ultracentrifugation in a denaturing solvent. Immunoglobulin subclasses in coho salmon serum should be considered, especially since the immunoelectrophoretic patterns of HMWIg from Lobb and Clem's (1981) studies are strikingly similar to those obtained here with purified coho serum immunoglobulin.

The purity of the rabbit antiserum specific for the coho tetrameric immunoglobulin was shown by its reaction with coho whole serum. Only one precipitin band was observed after immunoelectrophoresis and

other antibody populations were not apparent by this assay. If such proteins were present at relatively low concentrations, however, this assay might not have revealed their presence. Recalling again the work of Lobb and Clem (1981), they did not detect the sheepshead LMWIg by this same method because it was present in serum at 1/20th the concentration of the predominant HMWIg. The possible existence of other antibody populations in coho salmon serum was not ruled out by the experiments reported here.

Examination of the immunoelectrophoretic patterns obtained when mucus concentrates were assayed against rabbit serum specific for coho serum immunoglobulin are suggestive for the presence of mucus immunoglobulins. Two populations with minimal electrophoretic mobility are evidenced by the observance of two distinct bands near the mucus inoculated wells. The intensity of these bands, compared to the band obtained when the antiserum is diffused against coho whole serum, indicate that the mucus proteins were significantly heterologous or present at low concentrations. Both alternatives seem plausible since collection of mucus results in substantial dilution with water and unless the fish secretory antibody is homologous to its serum counterpart, some heterogenerity would be expected. The shape of the band nearest the well resembles the intense band observed when purified serum immunoglobulin, presumably tetrameric, is similarly immunoelectrophoresed. The more rapidly diffusing outer band is indicative of a smaller molecule and may represent an undescribed coho

immunoglobulin. Further studies with concentrates from large volumes of mucus would lead to more conclusive results.

These and other studies suggest that a level of complexity may exist in the serum and secretory constituents of the coho salmon immune system, and fish in general, that is worthy of further investigation. An understanding of the mechanisms of immune function is required to effectively approach the task of producing products for immunizating fish. A description of infectious processes in individual host-pathogen systems and parameters of the environment relating to these interactions are also needed to better control infectious diseases of fish.

SUMMARY AND CONCLUSIONS

1. Coho salmon tetrameric immunoglobulin was effectively absorbed in the blood of fish after it was intraperitoneally injected. At 12°C, absorption was complete by 8 h and reached a maximum level of 60% compared to the same material given intravenously.

2. The efficiency of absorption of intraperitoneally injected coho salmon tetrameric immunoglobulin was temperature related and less was absorbed at lower temperatures. Compared to intravenously injected fish, 37, 59 and 78% was absorbed at 6, 12 and 18°C, respectively, in intraperitoneally injected fish.

3. High levels of immunity to vibriosis were conferred by passive immunization with low titer coho salmon anti-*V. anguillarum* immunoglobulin partially purified by molecular sieve chromatography.

4. No protective immunity was conferred to previously unimmunized fish when they received serum or spleen cells from fish orally immunized against vibriosis.

5. Orally induced immunity against vibriosis in juvenile coho salmon is not a result of induced humoral antibodies.

6. Orally induced immunity against vibriosis in juvenile coho salmon is not a result of suspended bacterin that has leeched from the diet or fecal material.

7. Higher humoral agglutination titers to V. anguillarum were obtained with fish not anesthetized before immersion in bacterin. Equivalent levels of protection were induced in fish anesthetized or not anesthetized before immersion.

8. Mechanisms for uptake of bacterin by the immersion method of immunization are present in the head of juvenile coho salmon. Higher levels of immunity were obtained in fish whose posterior body was immersed and more important sites for uptake of antigen exist there.

9. Injection of the yolk material in an embryonated coho salmon egg can induce an immune response. This response indicates that immunological competency is attained before hatching or that immunogenic material can be retained within the fish or yolk until competency is attained.

10. Immersion of embryonated coho salmon eggs in V. anguillarum bacterin did not induce a long-lasting humoral response and a secondary humoral response could not be induced by a single injection of bacterin in the juveniles derived from these eggs.

11. Coho salmon alevins are not highly susceptible to infection by V. anguillarum. This resistance is lost as the yolk is absorbed and may therefore be associated with egg derived material.

12. An anamnestic response can be induced by injection of bacterin into the yolk material in an embryonated coho salmon egg near hatching followed by intraperitoneal injection of the fish derived from that egg.

13. Vibrio anguillarum can grow at 6°C and should be considered a psychrotroph.

14. The onset of serum agglutinin formation in juvenile coho salmon parenterally immunized against V. anguillarum is accelerated at high temperatures and delayed at low temperatures within temperature ranges the fish most frequently encounters.

15. Juvenile coho salmon can be infected with V. anguillarum by water-borne challenge at temperatures from 6 to 21°C. A shorter mean day to death and higher mortality occurred with increasing temperatures in this range.

16. Juvenile coho salmon anti-TNP serum antibodies obtained by affinity and molecular sieve chromatography were a homogeneous population as determined by immunoelectrophoresis and SDS-polyacrylamide gel electrophoresis.

17. Two components are present in juvenile coho salmon cutaneous mucus that share antigenic determinants with serum immunoglobulin.

18. Precipitin bands obtained by immunoelectrophoresis of mucus concentrate against rabbit antiserum specific for the serum immunoglobulin indicate that one of the mucus components may be similar to the serum immunoglobulin. An electrophoretically similar but more rapidly diffusing substance is also present and this may be another immunoglobulin population.

19. Orally induced immunity in juvenile coho salmon fed V. anguillarum bacterin is probably a result of antigenic stimulation of lymphoid tissues or cells in the gastrointestinal tract.

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