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Title: IMMUNE RESPONSES OF SALMONIDS: A) ORAL IMMUNIZATION AGAINST FLEXIBACTER COLUMNARIS, B) EFFECTS OF COMBINING ANTIGENS IN PARENTERALLY ADMINISTERED POLYVALENT VACCINES

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Juvenile coho salmon (Oncorhynchus kisutch) were orally immunized against the bacterium Flexibacter columnaris (formerly Chondrococcus columnaris), the causative agent of columnaris disease in fish, with a vaccine-food preparation containing formalin-killed cells. Fish immunized for 1, 2, 3, and 4 months were challenged with selected concentrations of F. columnaris by exposure in water. Protection from F. columnaris was first detected among fish immunized for 3 months and exposed to the lowest level of challenge used ($\sim 0.9 \times 10^5$ cells/ml). All challenges of fish immunized for 4 months indicated that the vaccine had induced an immune response against columnaris disease.

Two polyvalent vaccine studies were conducted to investigate the effect that combining of parenterally administered antigens had on immune responses of rainbow trout (Salmo gairdneri). Serum aglutinin titers were measured for indication of immune responses. In one study, formalin-killed cells of F. columnaris, Aeromonas salmonicida, and the causative agent (bacterium) of redmouth disease were mixed in all possible combinations and administered to different groups of fish. Responses of fish to antigens administered in the trivalent vaccine and the three divalent vaccines were monitored monthly for 3 months and compared with responses of fish injected with the corresponding monovalent vaccines. Comparisons indicated that responses to the combined antigens were not affected by either F. columnaris or the causative agent of redmouth disease. However, interference from A. salmonicida antigen suppressed responses to F. columnaris and the causative agent of redmouth disease. In the second study, formalin-killed cells of A. salmonicida, A. hydrophila, two strains of F. columnaris, and the causative agent of bacterial kidney disease were combined and injected into rainbow trout brookstock at the Oregon Wildlife Commission's Leaburg Trout Hatchery. Serum agglutinin titers of samples taken before vaccination and 3 and 12 months after vaccination were measured for each entigen. Excellent responses to A. salmonicida antigen and slight responses to the causative agent of bacterial kidney disease occurred. The fish did not respond to antigens of either strain of F. columnaris or A. hydrophila.

Immune Responses of Salmonids: A) Oral Immunization against *Flexibacter columnaris*, B) Effects of Combining Antigens in Parenterally Administered Polyvalent Vaccines

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IMMUNE RESPONSES OF SALMONIDS: A) ORAL IMMUNIZATION AGAINST FLEXIBACTER COLUMNARIS, B) EFFECTS OF COMBINING ANTIGENS IN PARENTERALLY ADMINISTERED POLYVALENT VACCINES

INTRODUCTION

Flexibacter columnaris (formerly Chondrococcus columnaris), the etiologic agent of columnaris disease, is a major fish pathogen occurring in freshwater rearing facilities and natural habitats. Outbreaks of columnaris disease occur frequently during warm weather when water temperatures exceed 14.4 C. Fish are more susceptible to the disease under hatchery conditions where they are sometimes stressed by handling and crowding.

Treatments with therapeutic agents are used in controlling F. columnaris. Chemical baths, antibiotics, and sulfonamides reduce the mortality rate when used during early stages of infection. However, once treatment is terminated, the disease can return to epizootic proportions if the organism is present in the water. Therefore, these agents are sometimes used frequently and for prolonged periods of time. Resistant strains of bacteria can develop in these circumstances making such control measures impractical.

Rather than treatments, preventative measures are preferred in controlling fish diseases. Induction of the immune response by a

vaccine is a prophylactic measure that can provide protection for a substantial period of time without drug resistant strains of pathogens developing.

A number of investigators have shown that poikilothermic animals are capable of responding to vaccines administered by the oral route. This method of immunization would appear to be the one of choice in fish hatcheries where controlled daily food rationing is possible and where handling involved in parenteral vaccination is undesirable. Since an oral vaccine that protects fish against Vibrio anguillarum has been developed (Fryer et al., 1970), other oral vaccines merit investigation.

The purpose of the oral immunization part of this research was to protect juvenile coho salmon (Oncorhynchus kisutch) against columnaris disease by the induction of an immune response. The objective was to develop an oral vaccine that might be perfected for mass immunization programs.

When antigens are combined in a polyvalent vaccine and injected into an animal, the immune response to one or more of the antigens can be affected by the combination. The response can be greater than, less than, or the same as one induced by a monovalent vaccine containing the same antigen. In considering a polyvalent vaccine, experiments are normally carried out comparing the immune responses to individual vaccine components when injected singly and

in combination. Possible side effects of the polyvalent vaccine can also be observed. Studies of this type can indicate whether or not antigens should be combined in a polyvalent vaccine.

The purpose of the polyvalent immunization part of this research was to evaluate the immune responses of rainbow trout (Salmo gairdneri) injected with multiple antigen vaccines. Bacterins made with the following organisms were used in this work: Aeromonas hydrophila, A. salmonicida, F. columnaris, the causative agent of redmouth disease, and the causative agent of bacterial kidney disease. The objective was to obtain information for development of immunization programs designed to protect fish from a variety of diseases.

LITERATURE REVIEW

Klontz and Anderson (1970) reviewed literature concerning oral immunization of fish. Some of the pertinent articles will be covered in this literature review.

Duff (1942) was the first to be successful with oral immunization of trout against furunculosis. He fed fish chloroform-killed cells of A. salmonicida incorporated into a ration consisting of 25% raw beef liver and 75% canned salmon. A 24% total mortality resulted among a group of fish immunized with 64-70 daily feedings when the fish were exposed to the organism by direct contact in water. The same challenge resulted in a 75% total mortality among non-immunized fish. The vaccinated fish also possessed higher antibody levels than non-vaccinated controls.

Post (1963, 1966) showed that antibodies against A. hydrophila could be produced by rainbow trout when fed an oral vaccine of heat-killed cells. In his experiment, adult fish received 1 mg (dry weight) of antigen every day for 262 days. All fish immunized by this method produced humoral antibody specific for A. hydrophila. Fifty percent of the vaccinated fish developed antibody levels that Post considered to be in the probable immune class. However, these fish did not possess significant protection compared to non-vaccinated controls when exposed to an intraperitoneally injected challenge of 1 LD_{90}.

Ross and Klontz (1965) fed phenol-killed cells of the causative agent of redmouth disease to yearling rainbow trout. Fish that received the vaccine for 360 days had a 31% total mortality when exposed to an intraperitoneally injected challenge of 40 LD_{90}. This challenge resulted in a 100% total mortality in the control group. However, Klontz and Anderson (1970) reported that subsequent experiments with this vaccine resulted in questionable protection from natural challenge in hatcheries.

Klontz and Anderson (1970) reported that an alum precipitated antigen (FSA), prepared from the water soluble portion of sonicated A. salmonicida cells, was non-toxic for fish and antigenically similar to the toxic extract of intact cells. Fish fed FSA at a level of 60 μg per day for 25 days developed humoral antibody. Fish immunized in this manner suffered no mortality upon exposure to a natural challenge of furunculosis. The fish were exposed 2 months after the last feeding of vaccine. Fifty-eight percent of a group of non-vaccinated fish died from furunculosis when exposed to the same challenge.

Overholser (1968) used the FSA antigen described above to orally immunize adult chinook salmon (Oncorhynchus tshawytscha). The total mortality of vaccinated fish was 0.7% following a natural challenge of A. salmonicida in a hatchery. Two non-immunized groups of fish suffered 37.0% and 22.2% total mortality due to furunculosis.

Fujihara (1971) reported the results of work with two F. columnaris oral vaccines. Heat-killed, whole cells were used in one preparation and sonicated cells in the other. The vaccines were suspended in saline and sprayed onto dry Oregon Moist Pellet (OMP). Both rations contained approximately 4×10^7 cells per gram of food. The heat-killed, whole-cell vaccine was fed to coho salmon daily for 7 weeks. The sonicated preparation was fed, according to the same schedule, to juvenile rainbow trout. Controls received normal OMP in both cases. The water source during these experiments was the Columbia River and was used as a natural challenge of F. columnaris for 10 weeks following the last feeding of vaccine food. Rainbow trout given the sonicated vaccine were not protected from columnaris disease. However, coho salmon fed the heat-killed, whole-cell vaccine were protected. This group of fish had an 8% total mortality compared to 48% among controls. Vaccinated coho also had higher antibody levels than the non-vaccinated controls.

Fryer et al. (1970) orally immunized chinook salmon (O. tshawytsha) against vibriosis. The vaccine consisted of a sonicated formalin treated preparation of V. anguillarum. Fish were immunized, while in freshwater, over a 14-day period. Each animal received an estimated dose of 300 μg of lyophilized preparation. The fish were allowed to remain in freshwater for 20 days after vaccination. They were then transferred to a saltwater rearing station

where V. anguillarum was known to be endemic. In the initial experiment 45% of the vaccinated fish and 98% of the non-vaccinated fish died from vibriosis. Greater differences between the mortality of vaccinated and non-vaccinated fish have resulted in recent experiments with the Vibrio vaccine.

Anderson and Ross (1972) tested the efficacy of four redmouth disease oral bacterins. Four methods of killing cells of the causative agent of redmouth disease were used in preparing the different antigens: incubation in 0.5% phenol solution for 24-48 hours; incubation in 3.0% chloroform solution for 4 days; sonication followed by incubation in 1.0% formalin solution for 12 hours; and incubation in 3.0% phenol solution for 12 hours followed by dialysis. Experimental fish received approximately 1,500 μg of vaccine in 6 weeks. Upon exposure to intraperitoneally injected challenges ranging from 4×10^3 to 4×10^8 live cells, all the vaccine-fed groups suffered lower total mortalities than the control groups. Fish immunized with the chloroform-treated preparation possessed the most protection.

Barr and Llewellyn-Jones (1952) reviewed the early literature concerning polyvalent vaccines. Their own experimental work dealing with antagonism and synergism between combined antigens indicated that there is no simple, general response to polyvalent vaccines. Some examples of the possible responses to polyvalent vaccines will be discussed in this literature review.

Combinations of antigens can induce immune responses equal to those induced by the individual antigens. Dillenberg (1962) showed that combining seven strains of Staphylococcus aureus and Staphylococcal Alpha-Toxoid in a vaccine had no adverse effect on the immune responses to the antigens. The polyvalent vaccine produced a full and lasting cure in 84% of 536 people with chronic cases of staphylococcal dermatoses. Similar results were attained using autogenous vaccines specific for the strain of the infection under treatment. Britton, Wepsic, and Moller (1968) showed that the immune responses in mice to Escherichia coli and sheep red blood cells were the same when suspensions of the two were administered separately or in combination.

Polyvalent vaccines can induce immune responses which are greater than those induced by monovalent vaccines. Maclean and Holt (1940) found that tetanus antitoxin titers were higher in a group of adult humans immunized with tetanus toxoid and typhoid-paratyphoid (T.A.B.) vaccine than in a group immunized with tetanus toxoid alone. Greenberg and Fleming (1947, 1948) showed that the Schick-negative rate was increased in guinea pigs injected with diphtheria toxoid if any of the following were administered at the same time: pertussis vaccine, tetanus toxoid, T.A.B. vaccine, or scarlet fever toxoid + pertussis vaccine. Kozlov (1967) found that rabbits injected with a combined smallpox-pertussis vaccine responded to each antigen

equally as well as rabbits injected with one of the antigens. However, after reimmunization, the agglutinating antibody titers against the pertussis antigen were higher in those animals which had received the combination.

Interference with antibody production can result from the combining of antigens into a polyvalent vaccine. Devoino (1959) immunized humans with two species of Shigella (flexneri and sonnei) which were known to induce the formation of humoral antibody. Agglutinating antibody titers increased for S. flexneri but not for S. sonnei during 4 months of observation. Shpigunov (1959) immunized pigs with smallpox, live attenuated brucellosis, and Gameleya polyvalent (against typhoid and paratyphi B) vaccines. He found that the brucellosis vaccine was less effective when administered in combination with both or either of the others. Kozinn, Wiener, and Burchall (1961) studied the effects of a six-strain Adenovirus vaccine in 104 children. By comparison of illness rates and serological studies, they concluded that the polyvalent vaccine was of little value. Antibody titers increased in the children for only three of the six strains of virus included in the vaccine. Previous work indicated that all six strains were capable of inducing the formation of antibodies.

MATERIALS AND METHODS

Flexibacter columnaris Oral Immunization Study

Antigen Preparation

A virulent strain (DD3-69) of *F. columnaris* was used in the production of antigen for oral immunization of coho salmon. The organism was recovered from the kidney of an adult spring chinook salmon at the Fish Commission of Oregon (F.C.O.) Dexter Dam holding pond in 1969. To minimize antigenic variation, lyophilized stocks of the isolate were prepared. Prior to lyophilization, the DD3-69 strain was passed through juvenile coho salmon seven times. This technique was used to increase the virulence of the isolate.

The following method was employed in preparing *F. columnaris* whole-cell antigen. Tubes of cytophage broth (Ordal and Pacha, 1967) were inoculated with lyophilized cell suspensions of the organism and incubated at room temperature (~ 23C) on a reciprocal shaker for 24 hours. The broth cultures were added to larger volumes (400-500 ml) of the same medium and incubated in the same manner. Three liters of turbid bacterial suspension were added to 30 liters of sterile cytophaga broth in a Fermacell Fermentor (New Brunswick Scientific Co., Inc., New Brunswick, NJ). Prior to sterilization of the medium in the fermentor, 150 ml of "Tween 80"

(Anacker and Ordal, 1955) was added to reduce clumping of the bacteria. The following conditions were controlled within the vessel for optimal growth: introduction of sterile air at a level of 28.32 l/min., mixing of contents at 200 rpm, temperature 25-28 C, and pH 7.2-7.4. A sterile, concentrated solution of buffered nutrients, equal to the amount used to prepare 30 l of cytophaga broth, was added to the fermacell culture after 12 hours incubation to increase the yield. After the incubation period, formalin was added to a final concentration of 0.3% and the mixture stirred for 45 min. to kill the cells. This was done after 24 hours incubation or upon formation of spheroplasts, whichever occurred first. Samples from the fermentor were checked for spheroplasts every hour during the last 6 hours of incubation. Samples were removed from all cultures and Gram stained for detection of spheroplasts and contaminants. Dead cells were collected by continuous centrifugation at 48,000 rpm in a Sharples Super Centrifuge. The majority of any spheroplasts present was carried away by the effluent because they are much lighter than vegetative F. columnaris cells. The cells were washed three times in sterile saline (0.85% NaCl in distilled water) and frozen in wet-packed form. Six batch cultures were grown with a combined yield of 230.5 g wet-packed cells.

Vaccine Preparation

Flexibacter columnaris antigen was incorporated into Oregon Moist Pellet (OMP) (Hublou, 1963) diet at the O.S.U. Seafoods Laboratory in Astoria, Oregon. A Waring blender was used to evenly suspend wet-packed cells in the liquid portion of the diet before any dry ingredients were added. An industrial mixer was used in blending the wet and dry portions of the vaccine-food preparation. The diet was then extruded through a 1/8 inch pellitizer and quick-frozen at -30 C. This ratio provided a vaccine concentration of 0.037 g (wet weight) antigen per 1 g of food. The number of cells in this dose was estimated according to weight. The dry weight of bacteria is approximately 25% of the wet weight (Meynell and Meynell, 1967). A milligram (dry weight) of a long gram-negative rod contains an estimated 3.1×10^9 cells (Meynell and Meynell, 1967) (see p. 25 for accuracy of estimating *F. columnaris* counts by dry weight). According to these estimations, the vaccine diet contained approximately 2.7×10^{10} cells per gram of food.

Immunization

Juvenile coho salmon (approximate weight 20 g) from Cascade Salmon Hatchery (F.C.O.) were used in the *F. columnaris* oral immunization experiment. Five hundred experimental fish were fed

vaccine-food while in a 463 l fiberglass tank. The waterflow was 7.6 l per minute and the water temperature was 12.2 C throughout the immunization period. Sixty-five g of frozen vaccine-food was administered daily during the first month of the study. This ration (~ 0.13 g per fish) provided an estimated 3.5×10^9 cells per day for each fish. Salmon were removed at monthly intervals for F. columnaris challenges. After each removal, the amount of vaccine-food preparation given the remaining fish was reduced to keep the estimated dosage constant. Normal OMP was supplemented into daily feedings during the last 2 months of the experiment when more than the vaccine-food ration was required by each animal. Control fish received normal OMP throughout the study, and were held under the same conditions as the experimental fish. Care was taken in rationing normal OMP in order that neither control nor experimental animals were favored nutritionally.

Challenge

Protection elicited by the F. columnaris oral vaccine was tested by exposing fish to live bacteria in water. Groups of 20 fish were challenged in 20 l of water containing various cell concentrations. This was done in 68.5 l fiberglass tanks with no waterflow. After 10 min. of challenge, the water was turned on allowing the tank to fill and the F. columnaris to be carried away in the effluent.

For optimal conditions of challenge, fish were removed from 12.2 C water and tempered to 17.2 C water. Work done in this laboratory indicated that the infectivity of F. columnaris at lower temperatures is inadequate for testing vaccines, while the virulence of the organism at high temperatures is likely to overcome immune responses in fish. In the tempering process, fish were removed from the large tanks they were held in during immunization and placed in 68.5 l tanks filled with 12.2 C water. The warmer water (17.2 C) was then introduced at a rate of 1.9 l per minute. Fish underwent this treatment 1 week prior to challenge. This provided a 7-day rest which possibly reduced susceptibility to F. columnaris due to stress from tempering. At the beginning of the 1-week rest, experimental fish were taken off the vaccine diet and fed normal OMP in order that antibodies and phagocytic cells would be free of antigen at the time of challenge.

Experimental and control fish were exposed to various concentrations of F. columnaris at monthly intervals throughout the experiment. Pathogen concentrations were varied by the addition of different amounts of F. columnaris to the water. All challenges for a given month were made with cells from the same bacterial suspension. This suspension was prepared by growing F. columnaris in 2 l of cytophage broth and diluting it with water to an optical density of 0.1 as measured by a spectrophotometer at 520 nm. The challenges used

in the experiment are listed in Table 1. Fish immunized for 1 month were exposed to challenges A-C listed in the table. After observing the results of these challenges, it was decided that lower concentrations of bacteria might be more effective in demonstrating protection possessed by vaccinated fish. Challenges B-E were used throughout the remainder of the study.

Table 1. Challenges used in the Flexibacter columnaris oral immunization study.

Challenge	Water (ml)	Bacterial suspension (0.1 O.D.) (ml)	Concentration[a/] (cells/ml)
A	19,600	400	1.8×10^6
B	19,800	200	0.9×10^6
C	19,900	100	4.5×10^5
D	19,960	40	1.8×10^5
E	19,980	20	0.9×10^5

[a/] Estimations made using standard plate count method with cytophaga agar.

Bacteriological Examination of Fish and Statistical Analyses of Mortality Data

Dead fish were removed from challenge tanks every 24 hours after the initial exposure to F. columnaris. The gills and kidney of each dead animal were cultured on cytophaga agar. The cultures were incubated at room temperature and examined daily for yellow, flat F. columnaris colonies with characteristic irregular edges.

Growth of such colonies, from either gill or kidney cultures was, considered a positive diagnosis of columnaris disease. All fish that survived challenge of F. columnaris were cultured in the same manner.

Differences in total mortality of control and experimental groups were tested by the chi-square method for contingency (Tate and Clelland, 1957).

Polyvalent Vaccine Studies

Antigen Preparation

Six antigens were prepared for use in the laboratory and hatchery polyvalent vaccine studies. When possible, virulent strains of bacteria were used, in case they possessed different antigenic determinants than less virulent strains. Five preparations were lyophilized and stored in vacuum desiccators until use. The sixth, which was prepared 5 days prior to vaccine preparation, was stored in saline at 4 C. The organisms used and the methods employed in preparing each antigen are described below.

Whole-cell antigen of the causative agent of bacterial kidney disease (Corynebacterium sp. ?) was prepared using an isolate originally recovered from the kidney of an adult spring chinook salmon at the Oregon Wildlife Commission (O. W. C.) Hood River Hatchery.

A lyophilized preparation of the organism was suspended in cold (4 C) cysteine serum broth (CSB) (formula of Ordal and Earp, 1956; modified by the use of calf serum in place of human blood) and inoculated onto a cysteine serum agar (CSA) slant (prepared in a 2 oz. prescription bottle). The culture was incubated at 18 C for 7-14 days and cells from the slant were suspended in cold CSB for use in inoculating three to four additional 2 oz. CSA slants. Subcultures were made following each incubation period until 100 slants of the organism were obtained. The cells from each culture were then suspended in formalized saline (0.2% formaldehyde solution in sterile saline) and stored for 12 hours at 4 C. The dead cells were washed three times with saline and stored in a concentrated suspension at 4 C. Before washing, samples of the cell suspension were Gram-stained or inoculated in Fluid Thioglycollate (Difco Laboratories, Inc., Detroit, Michigan). The stained cells were all short Gram-positive rods, characteristic of the kidney disease bacterium, and no growth occurred in the Thioglycollate. The suspension was thus assumed to be pure non-viable cells of the causative agent of bacterial kidney disease.

Cells used in determining serum agglutinin titers were grown in the same manner as the cells used in production of kidney disease antigen for vaccine use. The organism was killed by exposure to 60 C for 45 min., washed three times with saline, checked for

purity, and stored in concentrated suspension at 4 C. Heat rather than formalin was used in killing the cells to avoid autoagglutination during antibody titration. Prior to use, the concentrated suspension was diluted with saline to an optical density of 0.5 at 520 nm.

A virulent strain (As. Sil. 67) of A. salmonicida was used in preparing immunizing antigen. The organism was originally recovered from the kidney of a juvenile coho salmon that had died of furunculosis at the Siletz River Salmon Hatchery (F. C. O.) in 1967. Prior to lyophilization, the bacterium was passed twice in juvenile coho salmon and cultured on Furunculosis Agar (Difco) and Furunculosis broth (Spence et al., 1965).

Lyophilized A. salmonicida (As. SS70) whole-cell antigen for immunization purposes was obtained from Mr. L. R. Udey of this laboratory. The organism had been grown in Furunculosis broth in a Fermacell Fermentor. The cells were killed with formalin, washed three times with saline, and checked for purity before lyophilization.

Aeromonas salmonicida antigen used in measuring antibody titers was prepared from cells grown on Furunculosis Agar slants in 32 oz. prescription bottles. Because the virulent strain (As. SS70) autoagglutinated, a non-virulent strain (As. Sil. 67) isolated in 1967 from a coho salmon at the Siletz River Salmon Hatchery (F. C. O.), was used in titrations. The two isolates possess common antigenic

components which allows substitution of As. Sil. 67 in place of As. SS70 for measuring antibody titers. Each slant was inoculated with 5 ml of turbid broth culture and incubated at room temperature for 24 hours. The cells were killed with formalized saline, washed three times with normal saline, and stored at 4 C in concentrated form. Antigen used in antibody titrations was prepared by diluting the stock cell suspension with saline to an optical density of 0.85 at 520 nm.

The isolate used for production of redmouth disease antigen was recovered from the kidney of a juvenile rainbow trout during an epizootic at Gnat Creek Trout Hatchery (O. W. C.) in 1967. One hundred ml of nutrient broth was inoculated with a lyophilized preparation of the organism and incubated at room temperature on a reciprocal shaker for 24 hours. The broth culture was used to inoculate a 10 l carboy of the same medium which was incubated at room temperature for 24 hours and aerated by magnetic stirring. After incubation, cells were exposed to 0.5% formalin for 45 min. The dead cells were then collected and washed using the same methods previously described for harvesting F. columnaris cells grown in the Fermacell Fermentor.

Redmouth disease antigen used in antibody titrations was grown in smaller quantities (100-200 ml) of nutrient broth and aerated by shaking. The cells were killed with formalized saline, washed three

times with normal saline, and stored at 4 C in concentrated form. Antigen was prepared by diluting the suspension with saline to an optical density of 0.5 at 520 nm.

The isolate used for production of A. hydrophila antigen was recovered from the kidney of a shad (Alosa sapidissima) from the Coos River in 1966. Antigens used in immunological and serological procedures were produced using the same media and methods described in preparing the redmouth disease antigens.

Flexibacter columnaris used in injectable vaccines was grown and harvested using the same methods described in antigen production for the F. columnaris oral immunization study. In addition to the DD3-69 strain, an isolate with different antigenic composition was used (BH3-69). This organism was recovered from the kidney of an adult coho salmon at Bonneville Salmon Hatchery (F.C.O.) in 1969. Flexibacter columnaris used in antibody titrations was grown in small quantities (100-200 ml) of cytophaga broth. The optical density of the antigen was adjusted to 0.3 at 520 nm.

Laboratory Experiment with Combined Antigens

Vaccine Preparation. Seven vaccines were prepared for the polyvalent vaccine study at the Oregon State University Fish Disease Laboratory. Whole-cell antigens of F. columnaris (DD3-69),

A. salmonicida (As. Sil. 67) and the causative agent of redmouth disease were used in the experiment. Three monovalent vaccines, each containing one of the antigens, were produced. Three divalent vaccines, made with the three possible combinations of two antigens, were prepared. One vaccine contained all three antigens.

Vaccines were prepared by emulsifying antigens in equal amounts of saline and Freund complete adjuvant in a Waring blender, after the antigens had been homogeneously suspended in the saline fraction of the mixture. Each antigen was incorporated into the vaccine at a concentration of $\sim 4 \times 10^{10}$ cells per ml. Cell concentrations were estimated by dry weight (the antigens had been lyophilized in known volumes of saline for calculation of dry weights).

Experimental Animals. Yearling rainbow trout from Roaring River Trout Hatchery (O. W. C.) with an average weight of 50-75 g were used in the laboratory polyvalent vaccine study. Groups of 15 fish each were held in 463 l circular, fiberglass tanks throughout the study. Water with a constant temperature of 12.2 C was supplied to the tanks at ~ 3.8 l per minute. Oregon Moist Pellet was fed during the first 2 months of the experiment and Purina Trout Chow during the last 2 months.

Fish were marked with a cold brand in order that antibody levels in individual fish could be followed. In this method, fish were brought into contact for 1 to 2 seconds with a small brass brander that

was submerged in acetone and dry ice. The resulting brand was produced by release of melanin in the epidermis. Brands were placed in eight different areas on fish with or without adipose fin clips. This provided a distinct label for each individual within a group of 15 fish. All fish were rebranded every month because the brands had a tendency to disappear due to their location and growth of the fish.

Immunization. Seven groups of 15 fish each were immunized. Each group was administered one of the previously described vaccines. An eighth group was given a "placebo" of Freund complete adjuvant and saline. Injections were made into the peritoneal cavity just anterior to the pelvic fins. One ml syringes with 26 gauge needles were used in giving anesthetized fish 0.25 ml of vaccine. This amount contained ~ 1.0×10^{10} cells of each antigen included in the preparation. Fish were anesthetized in a dilute solution (~ 0.05%) of methyl pentynol.

Collection of Serum and Antibody Titration. Blood was obtained by cardiac puncture from each fish before vaccination in order to determine the existing background titers for each antigen. Blood samples were also taken 2 weeks, 1 month, 2 months, and 3 months after vaccination. In the cardiac puncture method, 21 gauge heparinized needles were inserted into the heart of aneathetized fish and 0.50 to 0.75 ml of blood was removed. The samples were placed in small, sterile culture tubes, incubated at room temperature for

1 hour, and stored for 8 hours at 4 C for clot retraction. Serum was removed from the clot after centrifugation for 10 min. at 1500 x g. Serum samples were then placed in small screw cap vials and frozen at -20 C. The vials were sealed with Parafilm (Marathon Products, Neenah, Wisconsin) prior to freezing to reduce evaporation.

Aeromonas salmonicida agglutinin antibody levels were measured using a microtiter agglutinating antibody titration (Vedros and Hill, 1966; Witlin, 1966). In this method, 50 µl quantities of fish sera were diluted by two-fold serial dilutions in standard "U" bottom microtiter plates. Fifty µl of A. salmonicida antigen was then added to each serum dilution and saline control. Agglutinin antibody levels of F. columnaris and the causative agent of redmouth disease were measured using a standard tube agglutination antibody titration (Kolmer, Spaulding, and Robinson, 1951). With both the microtiter agglutination and standard tube agglutination methods, antigen-antibody mixtures were incubated for 2 hours at room temperature followed by 24 hours at 4 C. Serums were titrated with each antigen in the vaccine that the sampled fish had received. Titers are reported as the reciprocal of the highest serum dilution that produced macroscopic agglutination (referred to as titer^{-1}).

Statistical Analyses. Statistical analyses were made comparing the mean titers^{-1} of serum samples from fish given one antigen (designated control) with mean titers^{-1} of serum samples from fish

given more than one antigen (designated treatment). Therefore, each of the three groups vaccinated with one antigen was termed control and compared with three treatment groups vaccinated with the same antigen plus one or two additional antigens. $Titer^{-1}$ values for individuals of each group were transformed to $\log_{10}$ and entered into a CDC 3300 computer. The computer performed a one-way analysis of variance with subsequent F-test to determine if there was a significant difference between any of the means. The significance of any differences between the means of the control and treatment $titers^{-1}$ was then determined by the method of Least Significant Difference (LSD) (Snedecor and Cochran, 1967). In this method, the mean log $titer^{-1}$ of each treatment group was subtracted from the mean log $titer^{-1}$ of the corresponding control group. This value was compared with the L. S. D. value which was supplied by the computer in the one-way analysis of variance. In cases where the L. S. D. value was smaller than the difference between the mean log $titer^{-1}$ of the treatment and control, the difference was significant at the 95% confidence level.

Polyvalent Vaccine Field Trial

Vaccine Preparation. A polyvalent vaccine containing five antigens was tested at the Leaburg Trout Hatchery (O. W. C.). Whole-cell antigen preparations of the following organisms were included in the vaccine: *F. columnaris* (DD3-69 and BH3-69), *A. salmonicida*

(As. Sil. 67), A. hydrophila, and the causative agent of bacterial kidney disease.

Antigen preparations were suspended in an emulsion of 50% saline-50% Freund complete adjuvant. A Petroff-Hauser counting chamber was used in estimating numbers of cells for each entigen except the causative agent of bacterial kidney disease. Counts were made on samples of the antigen preparations before lyophilization. The number of cells per milligram dry weight was then calculated. Counts were in close agreement ($3.0-4.6 \times 10^9$ cells per mg dry weight) with the estimation that there are 3.1×10^9 cells of a Gram-negative rod per milligram dry weight (Meynell and Meynell, 1967). The causative agent of bacterial kidney disease could not be counted using a counting chamber. Clumps of kidney disease bacteria formed during growth making the counting of individual cells inaccurate. Estimation by density using McFarland Nephelometer tubes was employed in this case. Bacterial kidney disease antigen was incorporated into the vaccine at a concentration of $\sim 2.3 \times 10^9$ cells per ml. The concentration of each of the remaining four antigens was $\sim 1.0 \times 10^{10}$ cells per ml.

Immunization. Young rainbow brood-stock weighing 400-500 g each were used in the polyvalent vaccine field trial. The temperature of the hatchery water supply at the time of immunization was 10.0 C.

(The water temperature ranged from 1.1 C to 12.2 C throughout the year.)

Five hundred fish received intraperitoneal injections just anterior to the pelvic fins. One ml syringes with 26 gauge needles were used in administering 1.0 ml of vaccine to each animal. This provided estimated doses of ~ 2.3×10^9 cells per fish for the bacterial kidney disease antigen and ~ 1.0×10^{10} cells per fish for each of the remaining four antigens. An additional 500 fish (controls) were given a 1.0 ml "placebo" of Freund complete adjuvant and saline. Experimental and control fish were marked with different pelvic fin clips and placed together in a pond with approximately 1000 uninjected fish. The capacity of the pond was such that stress conditions due to crowding did not arise during the experiment.

Collection of Serum and Antibody Titration. Prior to injection, 1.2-1.5 ml blood was obtained by cardiac puncture from each of 25 control and 25 experimental fish using 5 ml syringes with 18 gauge needles. A 0.0025% solution of ethyl-m-aminobenzoate methane sulfanate (Sigma Chemical Company, Saint Louis, Mo.) was used in anesthetizing fish. Serum was collected and agglutinin antibody levels against each antigen in the polyvalent vaccine determined, using the previously described microtiter and standard tube agglutination techniques. Titers are reported as the reciprocal of the highest serum dilution producing macroscopic agglutination.

Blood was drawn from 25 control and 25 experimental fish before vaccination, 3 months after vaccination, and 12 months after vaccination. Samples taken before vaccination were titrated to determine background antibody levels for each antigen in the vaccine. Three months post-vaccination was chosen as a sampling time when a good indication of the immune responses could be detected. This provided ample time for the fish to respond to each antigen. Peak antibody levels would have still been present at this time since the antigens were suspended in Freund complete adjuvant. The 3-month period between vaccination and sampling was from October 10, 1972 to January 10, 1973. During this period the water temperature at Leaburg Hatchery reached a low of 1.1 C. Paterson (1972) found that salmonids are capable of being immunized at 6.7 C. Temperature was, therefore, not considered as a factor that might hinder the immune responses between vaccination and sampling 3 months later. Three months after vaccination the fish responded well to only one antigen. Consequently, it was decided that samples would be taken 12 months after vaccination in order to determine if exposure to warm water would change the results.

RESULTS

Flexibacter columnaris Oral Immunization Study

Fish immunized for 1 month with F. columnaris oral vaccine were not protected from ~ 0.9×10^6 or ~ 1.8×10^6 F. columnaris cells/ml (Table 2). The total mortalities of vaccinated and non-vaccinated groups were not statistically different for both of these challenges (Table 2). Daily cumulative mortality graphs are nearly identical for the control and experimental groups of the above challenges (Figure 1). Fish vaccinated for 1 month experienced a significantly lower total mortality than non-vaccinated fish when exposed to ~ 4.5×10^5 cells/ml.

Fish fed the F. columnaris vaccine for 2 months possessed no protection from any of the test challenges (Table 2). The cumulative mortality curves of control and experimental groups are similar for each of the three highest F. columnaris concentrations (Figure 2). Experimental and control groups exposed to these three challenges experienced total mortalities within 5% of one another (Table 2). In the case of the lowest challenge (~ 0.9×10^5 cells/ml), the vaccinated fish had significantly greater loss from columnaris disease than the non-vaccinated fish (Table 2). This loss also began 4 days before that seen in the control group. Total mortalities of fish challenged at

Table 2. Statistical analyses of the total mortalities among Flexibacter columnaris orally vaccinated and non-vaccinated coho salmon (Oncorhynchus kisutch) following exposure to live Flexibacter columnaris.

Immunization time (months)	Challenge level (cells/ml)	Total mortality		Chi-square value
		vaccinated	non-vaccinated	
1	1.8×10^6	20[a]/20[b]	20/20	0.00
	0.9×10^6	13/20	15/20	0.48
	4.5×10^5	13/20	20/20	8.48*
2	0.9×10^6	17/20	17/20	0.00
	4.5×10^5	17/20	18/20	0.23
	1.8×10^5	18/20	17/20	0.23
	0.9×10^5	18/20	10/20	7.62*
3	0.9×10^6	10/20	14/20	1.67
	4.5×10^5	13/20	16/20	1.13
	1.8×10^5	14/20	19/20	1.49
	0.9×10^5	0/20	13/20	19.26*
4	0.9×10^6	1/20	7/20	5.63*
	4.5×10^5	1/20	15/20	20.42*
	1.8×10^5	7/20	16/20	8.29*
	0.9×10^5	0/20	5/20	5.71*

[a]Number of dead fish.

[b]Number of fish per group.

*Significantly different at the 95% confidence level.

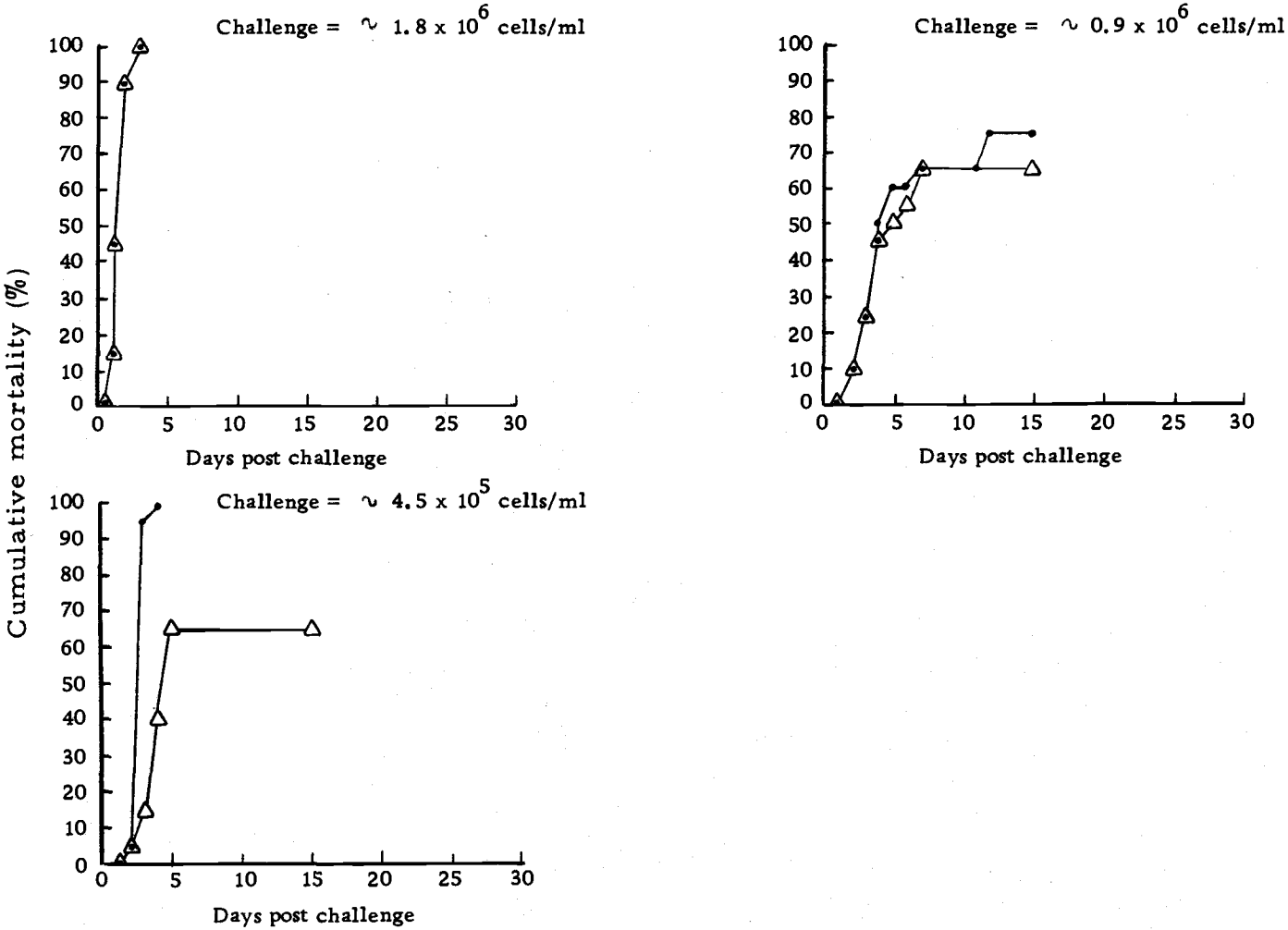

Figure 1. Results of Flexibacter columnaris challenges of juvenile coho salmon (Oncorhynchus kisutch) orally immunized for one month. △ = vaccinated, ● = non-vaccinated

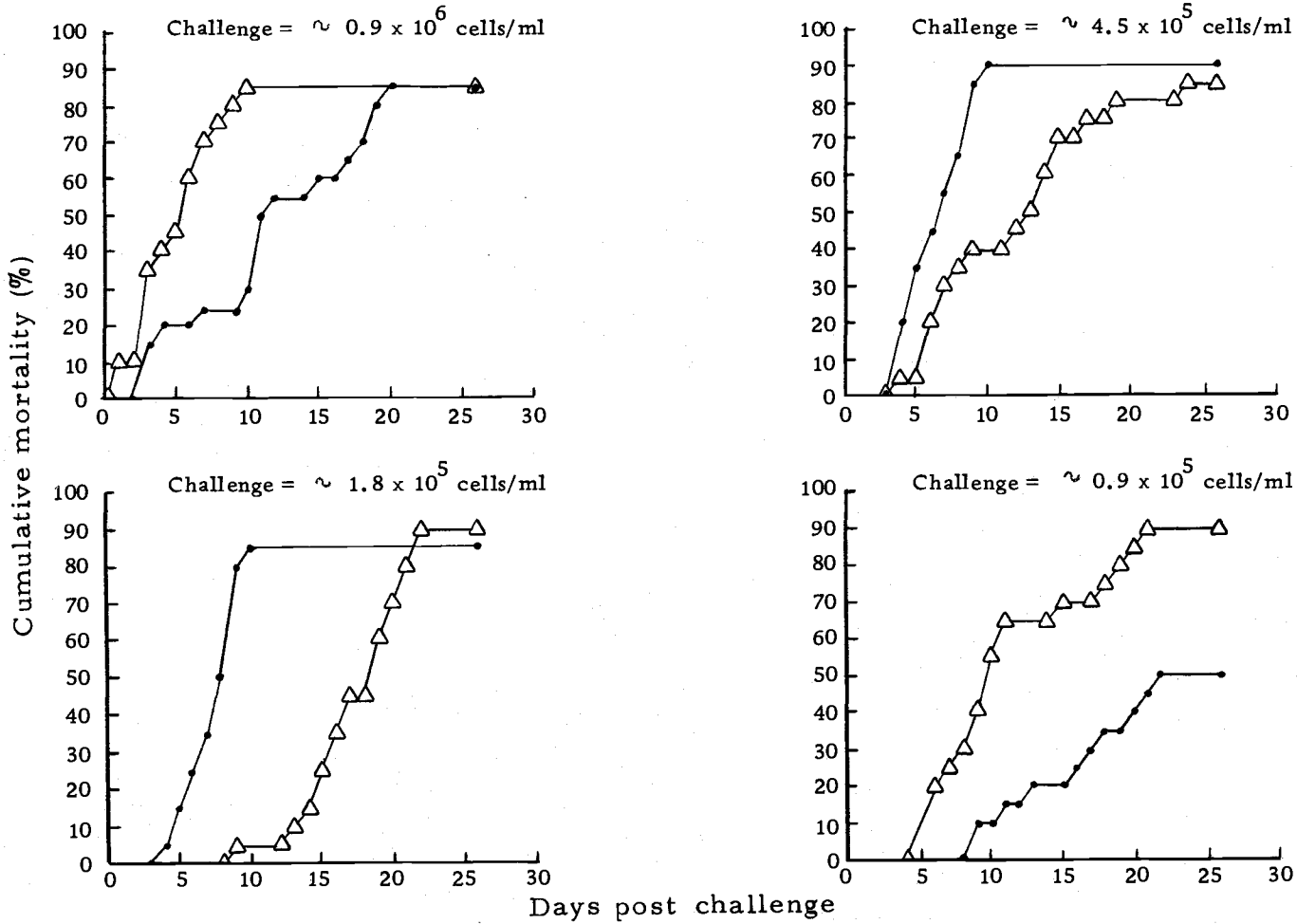

Figure 2. Results of Flexibacter columnaris challenges of juvenile coho salmon (Oncorhynchus kisutch) orally immunized for two months. Δ = vaccinated, ● = non-vaccinated

2 months post-vaccination did not peak and level off within 15 days of exposure as was the case the previous month. Extending the holding time after exposure from 15 to 26 days resulted in considerably more deaths due to columnaris disease.

All challenges at 3 months post-vaccination resulted in lower total mortalities among experimental groups than control groups (Table 2). However, this difference was not significant for groups exposed to the three highest concentrations of bacteria (Table 2). The lowest level of bacteria killed 65% of 20 control fish and none of the experimental fish. Extending the holding time after challenge from 26 to 34 days resulted in only one additional dead fish of one group (Figure 3).

All challenges at 4 months post-vaccination resulted in lower total mortalities among experimental groups than control groups. These differences were significant for each concentration of bacteria (Table 2). No experimental fish died when exposed to $\sim 0.9 \times 10^5$ cells/ml. Extending the holding period after challenge from 34 to 43 days resulted in no increase in mortality of any group (Figure 4).

Polyvalent Vaccine Studies

Laboratory Experiment with Combined Antigens

Serums from experimental animals sampled before vaccination

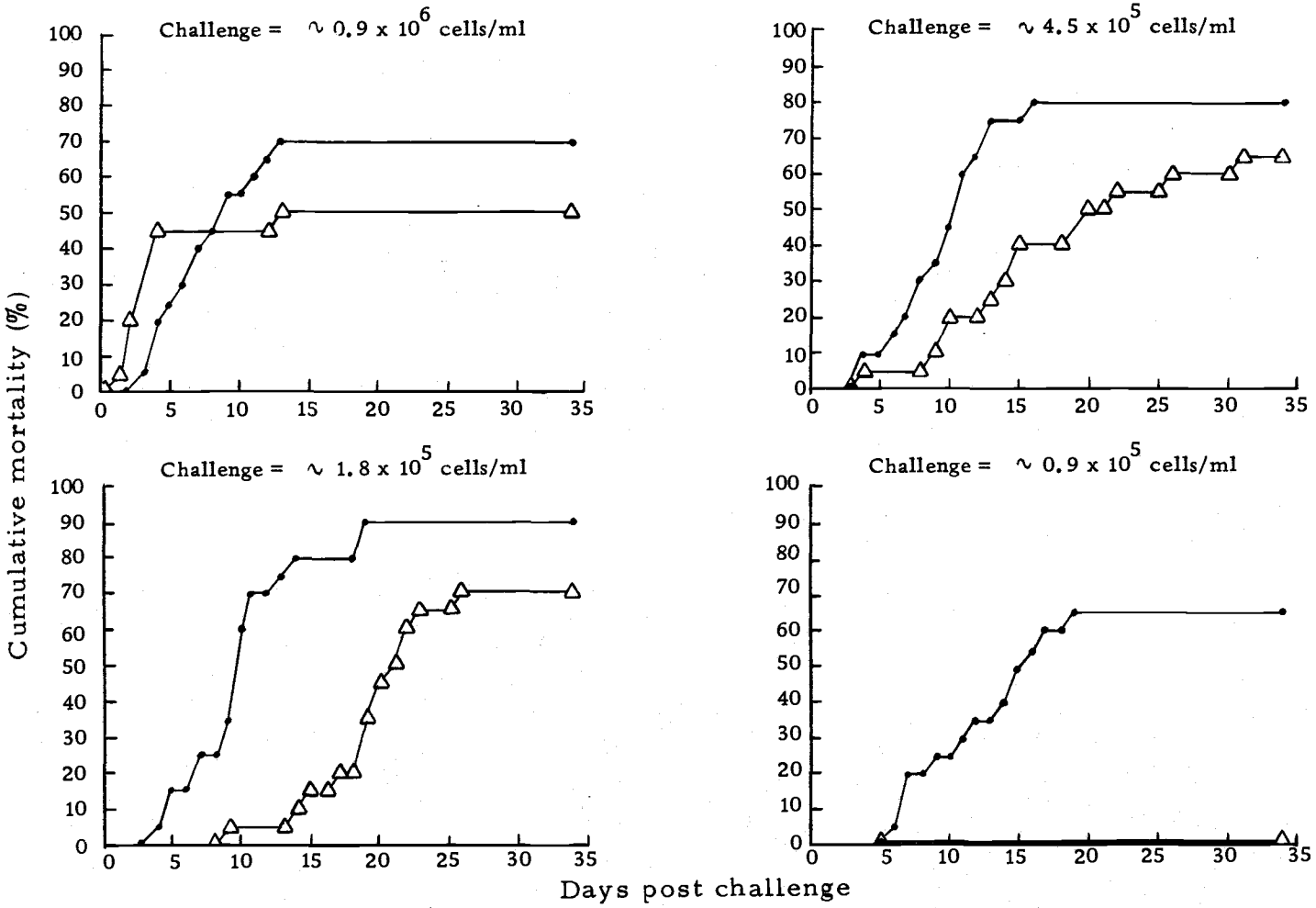

Figure 3. Results of Flexibacter columnaris challenges of juvenile coho salmon (Oncorhynchus kisutch) orally immunized for three months. Δ = vaccinated, ● = non-vaccinated

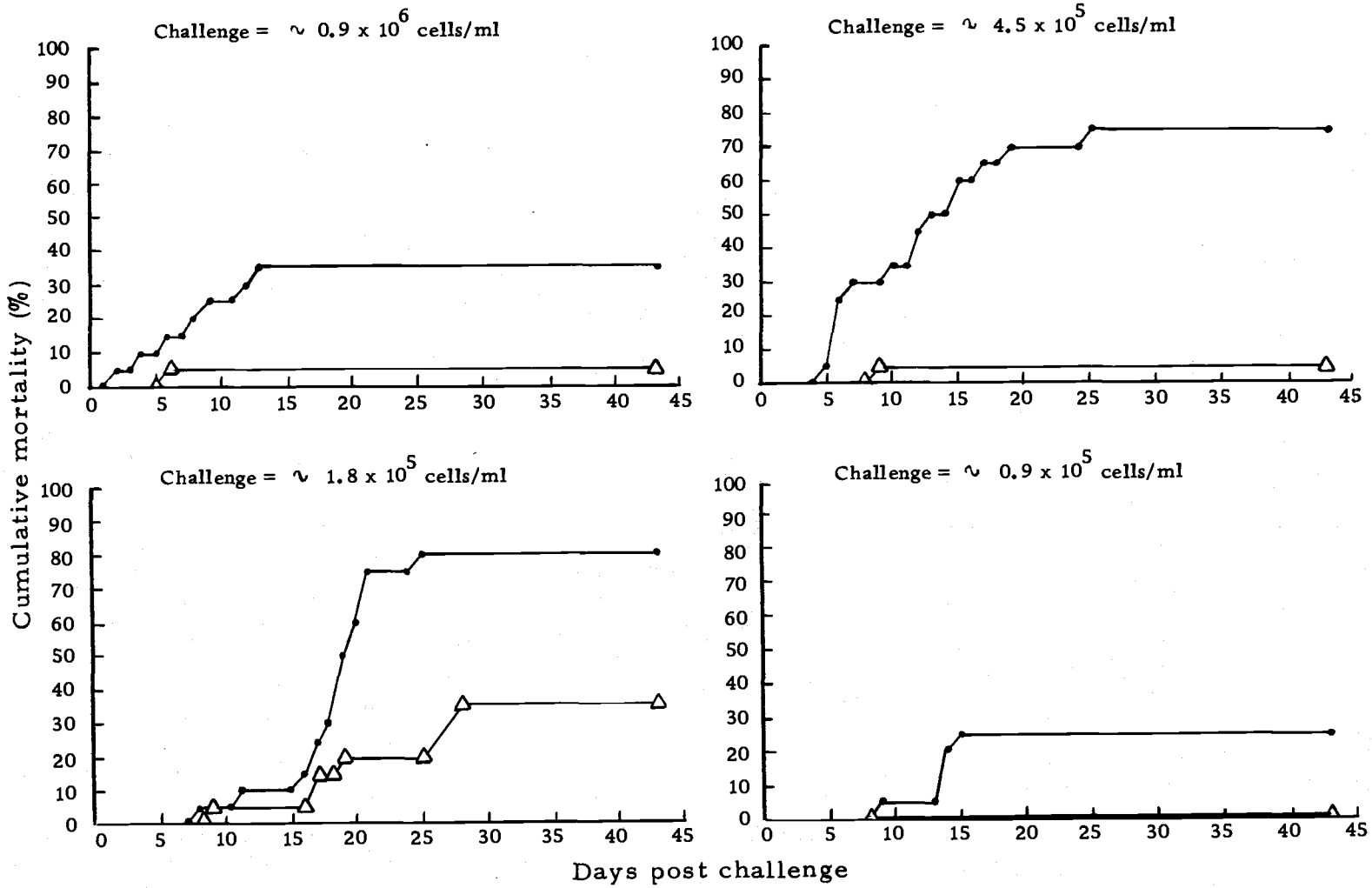

Figure 4. Results of Flexibacter columnaris challenges of juvenile coho salmon (Oncorhynchus kisutch) orally immunized for four months. ▲ = vaccinated, ● = non-vaccinated

produced no agglutination when titrated with F. columnaris or the redmouth disease bacterium. These titers were less than the highest concentration of serum tested, which was a 1:20 dilution. Slide agglutination tests (serum dilution = 1:2) run on randomly selected samples to detect small amounts of antibody also failed to show agglutinins against these two antigens. The titers were therefore assumed to be 0 for statistical analysis. A few fish in each group possessed background anti-A. salmonicida antibody titers^{-1} of 20 or 40 which resulted in low means for each group (Table 3).

There were no responses among fish in any group to F. columnaris antigen 2 weeks after vaccination (Table 3). Low levels of antibody specific for F. columnaris were detectable 1 month after injection among all groups of fish that had received the antigen. The anti-F. columnaris antibody levels were highest at this time among fish given the columnaris-redmouth vaccine.

Samples taken 2 months after vaccination showed that fish injected with the columnaris and the columnaris-redmouth vaccines responded significantly better to F. columnaris antigen than fish injected with columnaris-furnuculosis or columnaris-redmouth-furunculosis vaccines (Table 3).

Fish immunized with the columnaris vaccine continued to respond to the antigen through the 3-month sampling time. The anti-F. columnaris antibody titers decreased during this period among

Table 3. Geometric mean serum agglutinin titers^{-1} in rainbow trout (Salmo gairdneri) from a laboratory experiment with combined antigens.[a]

Sampling time	Antigens[b] in vaccine	Geometric mean agglutinin titers^{-1} for specified antigens		
		Flexibacter columnaris	Redmouth bacterium	Aeromonas salmonicida
Pre-vaccination	columnaris	0[c]	-[d]	-
	redmouth	-	0	-
	furnuculosis	-	-	5
	col. + red.	0	0	-
	col. + fur.	0	-	11
	red. + fur.	-	0	1
	col. + red. + fur.	0	0	2
	control	0	0	7
Two weeks post-vaccination	columnaris	0	-	-
	redmouth	-	0	-
	furunculosis	-	-	27
	col. + red.	0	0	-
	col. + fur.	0	-	24
	red. + fur.	-	0	23
	col. + red. + fur.	0	0	44
One month post-vaccination	columnaris	7	-	-
	redmouth	-	8	-
	furunculosis	-	-	469
	col. + red	23*	23*	-
	col. + fur.	16	-	579
	red. + fur.	-	1	368
	col. + red. + fur.	3	7	584
Two months post-vaccination	columnaris	32	-	-
	redmouth	-	81	-
	furunculosis	-	-	1,691
	col. + red.	65	93	-
	col. + fur.	13*	-	1,909
	red. + fur.	-	3*	1,739
	col. + red. + fur.	8*	27*	4,245
Three months post-vaccination	columnaris	107	-	-
	redmouth	-	306	-
	furnuculosis	-	-	20,789
	col. + red.	31*	335	-
	col. + fur.	7*	-	29,627
	red. + fur.	-	13*	14,763
	col. + red. + fur.	3*	31*	22,955
	control	0*	0*	5*

[a] Groups of 15 fish each were administered monovalent, divalent, or trivalent vaccines of Freund complete adjuvant plus formalin-killed cells of F. columnaris (DD3-69), A. salmonicida, and the causative agent of redmouth disease.

[b] col. = F. columnaris, red. = the causative agent of redmouth disease, fur. = A. salmonicida.

[c] No antibody detected at lowest serum dilution tested (1:2). A titer^{-1} of 0 was used in statistical analyses.

[d] Dash (-) indicates titrations were not run with antigens not present in vaccine.

* Significantly different at the 95% confidence level from mean titer^{-1} of fish injected with the corresponding monovalent vaccine as determined by one-way analysis of variance with subsequent F-test of Least Significant Difference.

fish injected with the other vaccines containing F. columnaris antigen. These decreased titers were significantly lower than those of serum samples from fish given the columnaris vaccine (Table 3).

There were no responses among fish in any group to redmouth disease antigen 2 weeks after vaccination (Table 3), but all groups of fish injected with redmouth antigen began to respond within 1 month of immunization. At this time, those fish that had received the columnaris-redmouth vaccine possessed the highest levels of antibody specific for the causative agent of redmouth disease.

Titrations of serum samples taken 2 and 3 months post-vaccination showed that fish given redmouth or columnaris-redmouth vaccine produced higher anti-redmouth disease antibody levels than fish injected with redmouth-furunculosis or columnaris-redmouth-furunculosis vaccines (Table 3).

All groups of fish that had received A. salmonicida antigen began responding to it within 2 weeks after vaccination (Table 3). There were no significant differences in the mean anti-A. salmonicida titers of groups injected with monovalent or polyvalent vaccines at any time during the study. Fish responded continually through the 3-month sampling time when peak titers were reached. Throughout the experiment, anti-A. salmonicida antibody levels were much higher than anti-F. columnaris or anti-redmouth disease antibody levels (Table 3).

Control fish injected with Freund complete adjuvant and saline were sampled throughout the study. Serum samples taken from these fish before vaccination and 3 months after vaccination showed no detectable antibody for the causative agents of redmouth or columnaris disease (Table 3). Background antibody titers did exist for A. salmonicida but remained unchanged after 3 months (Table 3). Antibodies specific for F. columnaris or the causative agent of redmouth disease were undetectable among control fish 3 months after injection of adjuvant and saline. Titrations were not run on other serum samples from this group of fish since it was evident that nothing at the laboratory was causing fish to produce antibodies specific for the antigens used in the experiment. It was assumed that increases in antibody titers of fish injected with vaccines were due solely to the presence of antigens in the vaccines.

Polyvalent Vaccine Field Trial

Serum samples taken before injection of the polyvalent vaccine were titrated with each of the five antigens in the vaccine (F. columnaris DD3-69 and BH3-69, A. hydrophila, A. salmonicida, and the causative agent of bacterial kidney disease). No background titers were present in any of 50 serum samples for either strain of F. columnaris, while titers did exist for the remaining three antigens. Geometric mean titers^{-1} of all serum samples from this study are listed in Table 4.

Table 4. Geometric mean agglutinin titers^{-1} of serum samples from rainbow trout (Salmo gairdneri) in a polyvalent vaccine field trial.[a]

Antigen titrated	Time of sampling				
	Pre-vaccination (N=50)	3 Months post-vaccination		12 Months post-vaccination	
		control (N=25)	experimental (N=25)	control (N=25)	experimental (N=25)
Flexibacter columnaris					
DD3-69	0[b]	0	0	0	0
BH3-69	0	0	0	0	0
Aeromonas hydrophila	24	15	14	10	13
Aeromonas salmonicida	43	38	1008*	14	338*
Causative agent of bacterial kidney disease	55	44	78*	6	6

[a] Young brood fish weighing 400-500 g were injected intraperitoneally with a polyvalent vaccine containing the following antigens: F. columnaris DD3-69 and BH3-69, A. hydrophila, A. salmonicida, and the causative agent of bacterial kidney disease.

[b] No antibody detected at lowest serum dilution tested (1:2). A titer^{-1} of 0 was used in statistical analyses.

* Mean titers^{-1} of control and experimental fish significantly different at the 95% confidence level as determined by the t-test.

Antibodies specific for the two strains of F. columnaris were undetectable in control fish throughout the experiment (Table 4). Mean titers for the remaining three antigens dropped slightly among non-vaccinated fish during the first 3 months of the study and continued to drop through 12 months post-vaccination when the experiment was terminated.

Vaccinated fish responded to only two of the five antigens in the vaccine. Anti-F. columnaris antibody levels in these fish were undetectable throughout the experiment. Mean anti-A. hydrophila antibody titers were slightly lower among vaccinated fish sampled 3 and 12 months post-vaccination than those sampled before vaccination. The mean anti-A. salmonicida antibody titer among experimental fish rose sharply during the first 3 months after vaccination then dropped slightly during the last 9 months of the study. Antibodies specific for the causative agent of bacterial kidney disease increased among vaccinated fish during the first 3 months of the experiment and then decreased during the next 9 months to levels lower than the mean background titer.

DISCUSSION

Flexibacter columnaris Oral Immunization Study

Fish immunized for 1 or 2 months were not protected from any F. columnaris challenges. Fish immunized for 1 month and challenged with ~4.5×10^5 F. columnaris cells/ml did experience a significantly lower total mortality than non-vaccinated fish exposed to the same challenge. However, this cannot be interpreted as protection because there was no significant difference in the total mortalities of vaccinated and non-vaccinated fish exposed to the above challenge in the following 2 months of the experiment.

The two occasions when significant differences in total mortality between vaccinated and non-vaccinated fish occurred during the first 2 months of the study may have been due to cross infection within a tank. Dead fish were removed from the tanks every 24 hours. If a fish died just after the time of collection, F. columnaris cells could have been sloughed from the dead fish for nearly 24 hours. While most of these bacteria were probably carried away in the effluent, at least some may have come into contact with live fish and could have affected the total mortality within a group. Fujihara and Nakatani (1971) found that effluent from troughs holding rainbow trout contained up to 190 F. columnaris cells/ml while water entering the troughs

contained less than one F. columnaris cell/ml. Cross infection could also explain why groups exposed to lower challenge levels sometimes suffered greater total mortality than groups exposed to higher challenge levels.

Protection afforded by the vaccine among fish immunized for 3 months was apparently overcome by the three highest challenges. There was a definite difference in F. columnaris susceptibility between fish immunized for 3 months and non-immunized fish when both were exposed to the lowest challenge level. Both biological and statistical aspects of the results were significant here in indicating that the vaccine had induced an immune response. It is improbable that 20/20 fish could have survived exposure to $\sim 0.9 \times 10^5$ F. columnaris cells/ml without possessing protection against the organism. This challenge level is much greater than concentrations of F. columnaris which fish are exposed to naturally. Fujihara and Hungate (1971) isolated less than 5 F. columnaris cells/ml during periodic sampling of water from four fish ladders in the Columbia River. Water downstream from these ladders contained less than 1 organism/ml. These cell counts are probably close to maximum numbers of F. columnaris that fish normally encounter in the Columbia River since Fujihara and Hungate (1971) sampled during the warm months of the year when peak concentrations of this organism

are present. Stevens (personal communication) found similar concentrations of F. columnaris in Columbia River fish ladders.

Significant differences in total mortality of vaccinated and non-vaccinated fish resulting at every challenge level in the last month of the study indicated that coho salmon could be protected from high levels of F. columnaris when given the vaccine for 4 months. The low total mortality of non-vaccinated fish exposed to $\sim 0.9 \times 10^6$ and $\sim 0.9 \times 10^5$ cells/ml may raise some doubt as to whether or not the vaccinated fish were actually protected from significant levels of virulent F. columnaris. The protection is more easily recognized when examining the combined total mortalities of vaccinated fish exposed to the lowest challenge ($\sim 0.9 \times 10^5$ cells/ml) at 3 months vaccination and to all challenges ($\sim 0.9 \times 10^5$-0.9×10^6 cells/ml) at 4 months vaccination compared to the combined total mortalities of non-vaccinated fish exposed to these challenges. In this comparison, the combined total mortality of the five vaccinated groups was 9/100 while that of the five non-vaccinated groups was 56/100. Examining the results of these five challenges for the interpretation of protection is valid because 1) significant responses began at 3 months vaccination with the three highest challenges overcoming the immunity, 2) responses continued during the fourth month of vaccination resulting in increased immunity that could be detected at each challenge.

Challenges with high levels of bacteria affected the number of daily vaccine feedings required for protection. Work done in this laboratory indicated that F. columnaris is a fairly weak antigen which may be another reason for a long immunization period. Successful oral immunization of fish against other pathogens has required less time in some cases and more time in others than required in this study. Duff (1942) orally immunized cutthroat trout (Salmo clarki) against A. salmonicida with 64 daily vaccine feedings. Rohovec (1974) immunized chinook salmon against vibriosis with 15 daily feedings of vaccine. Post (1966) reported that the number of rainbow trout in the probable immune class reached a peak in his study after 262 daily feedings of A. hydrophila antigen.

The main difference of this work from the successful immunization of coho salmon against columnaris disease by Fujihara and Nakatani (1971) is that this study employed higher levels of challenge which required more protection. Fujihara and Nakatani (1971) fed a diet containing 4×10^7 cells/g food for 49 days and exposed their fish to a constant flow of Columbia River water which contained less than 1 F. columnaris cell/ml. In the present study, $\sim 2.7 \times 10^{10}$ cells/g food was fed for 122 days and the fish were exposed to a short period of challenge with as many as 1.6×10^6 F. columnaris cells/ml. Thus, protection of coho salmon from extremely high concentrations of F. columnaris was achieved by administering a diet containing 900

times more vaccine for 73 days longer than the immunization procedure of Fujihara and Nakatani (1971). It is probable that a higher level of protection would have resulted in fish immunized in the present study if a challenge similar to Fujihara and Nakatani's (1971) had been used.

The fact that coho salmon could be immunized and withstand exposure to unnaturally high levels of F. columnaris is of special interest in considering vaccination of fish that would be subjected to stress factors. Since stresses, such as low oxygen levels and high temperatures, are present in virtually every habitat where columnaris disease is a threat, strong protection would be essential if such a vaccine were to be put into practical use. An oral columnaris vaccine may not be economically feasible at present because the growth dynamics of F. columnaris in cytophaga broth are such that substantial amounts of antigen cannot be easily produced. Since increasing the concentrations of the components in cytophaga broth results in higher yields of F. columnaris (unpublished work done in this laboratory), development of a more suitable media for this organism may be possible. This would be necessary for large scale production of the vaccine.

Laboratory Experiment with Combined Antigens

Undetectable and low background titers observed among rainbow trout in this experiment indicated that these fish had had little or no

recent contact with epizootic levels of the antigens employed in the vaccines. This allowed a more accurate evaluation of the responses than if the fish had possessed high background titers. The lack of detectable amounts of anti-F. columnaris antibody is not unusual among hatchery stocks in Oregon. Holt and Sanders (personal communication) found very low or undetectable levels of antibody specific for F. columnaris among salmonids in Oregon. In contrast to background anti-A. salmonicida antibody levels found in the present investigation, Paterson (1972) reported relatively high (10-160) anti-A. salmonicida background titers^{-1} among coho salmon that had been exposed to furunculosis. Frost (1968) and Krantz (1963) have also reported high anti-A. salmonicida antibody titers in non-vaccinated salmonids. No literature is available concerning levels of anti-redmouth antibody acquired through natural exposure to the causative agent of this disease.

In comparing the mean titers of fish immunized with vaccines containing more than one antigen to the mean titers of fish immunized with monovalent vaccines, it is evident that antigen competition occurred. Aeromonas salmonicida antigen inhibited the responses of rainbow trout to F. columnaris and the causative agent of redmouth disease. The antagonistic reaction of A. salmonicida was strong enough to suppress the responses to both of the other antigens when

fish were immunized with the columnaris-redmouth-furunculosis vaccine.

Results of this study indicated that F. columnaris antigen was not competitive with A. salmonicida or the causative agent of redmouth disease.

The causative agent of redmouth disease had no effect on responses to A. salmonicida antigen and little effect on responses to F. columnaris antigen. Statistically, the combination of the causative agent of redmouth disease and F. columnaris antigen produced an antagonistic response to F. columnaris but not to the redmouth antigen during the last month of the study. However, it should be noted that the peak mean anti-F. columnaris titer of the group immunized with the columnaris-redmouth vaccine was not significantly different from the peak mean anti-F. columnaris titer of the group immunized with the columnaris vaccine (these peaks were reached at different times). Therefore, the apparent difference between these groups does not indicate that the antigens were competitive.

Polyvalent Vaccine Field Trial

Because water temperatures of the McKenzie River never reach 14.4 C, there have been no problems with columnaris disease at Leaburg Hatchery. This partially explains the absence of background antibody levels for the two F. columnaris isolates among the rainbow

trout broodstock in this experiment. The low anti-A. hydrophila antibody levels were expected since this organism is ubiquitous among freshwater fishes but has not been responsible for any epizootics at this rearing facility. There have been losses due to kidney disease and furunculosis among the chinook salmon at Leaburg Hatchery which is probably why mean background antibody titers were high for the causative agents of these diseases. Populations of adult salmon and trout in the McKenzie River above the hatchery water intake also could have contributed sublethal levels of kidney disease and furunculosis to the water which may have been responsible for the anti-furunculosis and anti-kidney disease background antibody levels. Reports of anti-A. salmonicida background titers in salmonids are discussed in the section headed Laboratory Polyvalent Vaccine Study. Bullock (personal communication) found a mean anti-kidney disease antibody titer^{-1} of 48 among adult brook trout (Salvelinus fontinalis) exposed to bacterial kidney disease. Unexposed adult brook trout had a mean titer^{-1} of 16 in his study.

Slight responses to the kidney disease antigen and the absence of responses to the F. columnaris and A. hydrophila antigens indicated that competition from A. salmonicida antigen also occurred in this experiment. At 3 months post-vaccination, antibody production for the causative agent of bacterial kidney disease was partially suppressed by competition with A. salmonicida antigen. Evelyn (1971) reported

that sockeye salmon (Oncorhynchus nerka) were capable of producing anti-kidney disease antibody titers^{-1} of 10, 240. Although the present study was not with sockeye salmon, the fact that salmonids with substantial anti-kidney disease background levels were used indicated that, in the absence of antigen competition, the fish probably would have responded better to the kidney disease antigen. The fact that responses to the causative agent of bacterial kidney disease were not totally suppressed at 3 months post-vaccination showed that competition with A. salmonicida varied with antigens injected into rainbow trout, and suggests that there may be antigens which would not compete with A. salmonicida. For this reason, an antagonistic effect in salmonids should not be assumed for antigens not tested with A. salmonicida.

Results at 12 months post-vaccination indicated that the vaccinated fish were still responding well to A. salmonicida but had stopped responding to the kidney disease antigen. Evelyn (1971) found that sockeye salmon produced high anti-kidney disease antibody titers at 3 months post-vaccination but that antibody levels among fish in his study had dropped significantly by 16 months post-vaccination. This suggests that the low anti-kidney disease antibody titers in the present study at 12 months post-vaccination (compared to those at 3 months post-vaccination) may have been a result of decreased response due to the time lapse after vaccination rather than antigen competition by

A. salmonicida. The mean anti-A. salmonicida titer^{-1} of 338 at 12 months post-vaccination is not unusually high for fish vaccinated with a Freund adjuvant suspension of this antigen. Krantz et al. (1963) and Paterson (1972) also reported high anti-A. salmonicida antibody titers long after vaccinations of this type.

Antibody titrations of samples taken 12 months after vaccination indicated that the vaccinated fish were unable to produce higher levels of antibody after exposure to warm water (12.2 C) than they did during the first 3 months of the study when they were exposed to cold water (as low as 1.1 C). This supports the finding by Paterson (1971) that salmonids are capable of an immune response at low temperatures. However, it is possible that slightly higher titers for the five bacterins would have been present 3 months after vaccination if the hatchery water had been a constant temperature of 12.2 C.

There is too little known about antigen competition to do more than speculate about the mechanism by which A. salmonicida reacted antagonistically with other antigens used in the two polyvalent vaccine studies in this research. Of the three theories concerning antigen competition, referred to by Hanna and Peters (1969), the antagonistic responses resulting with A. salmonicida antigen are most in accordance with the theory of Schecter (1967). Schecter (1967) explains competition between simultaneously injected antigens as the result of preferential binding to a limited number of multipotential antibody

forming cells. The other two theories referred to by Hanna and Peters (Radovich and Talmage, 1967; Eidinger, Khan, and Millar, 1968) require injection of antigens at different times which allows the initially injected antigen to act as a stimulus for competition. Pross and Eidinger (1974) discuss nine theories of antigen competition and cite numerous reports of data supporting each theory. Six of these theories are based on administering antigens at different times. The remaining three theories are possible mechanisms for competition in fish by A. salmonicida simultaneously administered with antigens used in the present study. These three theories are as follows: 1) competition of antigens for receptor cells occurring in limited frequency, 2) interference between thymus cell-derived helper molecules on the surface of macrophages which present antigen to specific B cells, and 3) competition between antigens bearing cross-reactive determinants.

Competition resulting in the two polyvalent vaccine studies indicated that A. salmonicida should not be included in a parenterally administered vaccine for rainbow trout with the following antigens: F. columnaris, A. hydrophila, the causative agent of bacterial kidney disease, and the causative agent of redmouth disease. This does not preclude the potential for polyvalent vaccines in salmonids.

SUMMARY AND CONCLUSIONS

Coho salmon were orally immunized against F. columnaris with a vaccine-food preparation containing ~ 2.7×10^{10} formalin-killed bacteria/g. Protection began after 3 months immunization and was detectable when fish were challenged with ~ 0.9×10^{5} F. columnaris cells/ml. After 4 months immunization, fish were protected from bacterial concentrations as high as ~ 0.9×10^{6} cells/ml.

Work done with parenterally administered polyvalent vaccines indicated that A. salmonicida antigen significantly suppressed immune responses of rainbow trout to the causative agents of redmouth and columnaris disease when combined with either or both of these antigens. Fish injected with a trivalent vaccine containing these antigens produced serum agglutinin titers for A. salmonicida equal to titers produced by fish injected with either a monovalent furunculosis vaccine or a divalent furunculosis vaccine containing F. columnaris or redmouth disease antigen.

Bacterins of F. columnaris and the causative agent of redmouth disease did not react antagonistically when both were combined in Freund complete adjuvant and injected intraperitoneally into yearling rainbow trout.

A polyvalent vaccine field trial with rainbow trout broodstock resulted in excellent immune responses to A. salmonicida, slight

responses to the causative agent of bacterial kidney disease, and no responses to A. hydrophila or two strains of F. columnaris when all five formalin-killed antigens were suspended in Freund complete adjuvant and injected intraperitoneally.

Results from the two polyvalent vaccine experiments indicated that A. salmonicida should not be included in a parenterally administered vaccine for rainbow trout with any of the following antigens: F. columnaris, A. hydrophila, the causative agent of bacterial kidney disease, and the causative agent of redmouth disease.

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