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HUMORAL FACTORS IMPORTANT IN RESISTANCE OF SALMONID FISH TO BACTERIAL DISEASE. I. SERUM ANTIBODY PROTECTION OF RAINBOW TROUT (*SALMO GAIRDNERI*) AGAINST VIBRIOSIS

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ABSTRACT

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Serum antibody is shown by passive immunization and absorption experiments to be an important component of defense mechanisms against experimentally induced vibriosis in rainbow trout (*Salmo gairdneri*). Antigen from heat-killed *Vibrio anguillarum* injected into trout with Freund's complete adjuvant induced much higher agglutinin titers than formalin-killed bacteria with or without adjuvant, or heat-killed bacteria without adjuvant. Passively transferred trout anti-*Vibrio anguillarum* serum provided protection against experimental vibriosis in trout for more than 2 months. Passively transferred anti-*Vibrio anguillarum* rabbit serum also protected trout.

INTRODUCTION

With the advent of experimental aquaculture and the promise of a substantial saltwater salmon rearing industry, the disease problems of salmonid fishes are of increasing concern. Drug therapy and prophylactic procedures such as the avoidance of predisposing environmental conditions are successful to some degree, but these methods alone are not sufficient for controlling disease in hatcheries and other areas where salmon and trout are held in high concentrations.

A number of studies have been made of immune responses in salmonid fishes, and attempts have been made to use immunological methods for disease prevention and control. Duff (1942), working with cutthroat trout (*Salmo clarki*) and the fish pathogen *Aeromonas salmonicida*, was the first to show that fish could successfully be orally immunized; his vaccine consisted of the chloroform-killed pathogen. Others subsequently tested bacterins for preventing fish diseases, with various degrees of success, using both parenteral and oral routes of immunization (Krantz et al., 1963; Post, 1963; Hayashi et al.,

1964; Ross and Klontz, 1965; Snieszko, 1970; Fujihara and Nakatani, 1971; Fryer et al., 1971; Anderson and Ross, 1972). Immune responses of salmonid fishes were also investigated from the perspective of phylogenetic relationships and for possible use in disease control (Klontz, 1959; Post, 1963; Hodgins et al., 1965, 1967; Ridgway et al., 1966; Chiller et al., 1969 a and b; Evelyn, 1971; Cisar, 1972; Roubal et al., 1974).

The purpose of the present work was to advance the understanding of the nature of humoral protective mechanisms involved in the immune response of salmonid fishes to *Vibrio anguillarum*. This bacterium was chosen for study because it is responsible for substantial mortalities of salmonid fishes held in pens in salt water in Puget Sound and other areas of the Pacific Northwest (Novotny, 1972).

MATERIALS AND METHODS

Experimental animals

Twenty-six 700—900-g rainbow trout (*Salmo gairdneri*), held at 14—16°C in fresh water at the Northwest Fisheries Center, Seattle, Washington, were used to produce antisera against *V. anguillarum* and *Escherichia coli*. The protective effect of these antisera, and of a xenogeneic anti-*V. anguillarum* serum prepared in three female New Zealand white rabbits, was studied using passively immunized juvenile rainbow trout (*S. gairdneri*) (30—75 g) obtained from the Chambers Creek Hatchery, Washington State Department of Game.

During handling, the trout were anesthetized with tricaine methane sulfonate (10 ppm) to minimize stress, and they were all tested prior to immunization and found to be free of anti-*V. anguillarum* agglutinins.

Microorganisms

A virulent strain of *V. anguillarum* (coded 700-23) was used in this study; it was generously provided by A.J. Ross of the Western Fish Disease Laboratory of the U.S. Fish and Wildlife Service, Seattle, Washington. The bacterium, originally isolated from diseased salmon at the National Marine Fisheries Service (NMFS) Saltwater Experiment Station at Manchester, Washington, was grown in the laboratory at 20°C on trypticase soy agar with 1.5% NaCl added and maintained on slants of the same medium under paraffin oil. The bacterium was biochemically characterized as *V. anguillarum* by Dr Bruce Braaten of the Northwest Fisheries Center, Seattle. Further confirmation of identity by DNA homology analysis was made by Dr Erling Ordal of the Department of Microbiology, University of Washington School of Medicine, Seattle.

To examine whether specific anti-*V. anguillarum* antibody was involved in protecting trout against vibriosis, the protective effect of an antiserum prepared against *E. coli* was also investigated. The strain of *E. coli* used was

obtained from the Department of Microbiology at the University of Washington and serologically was not cross-reactive with *V. anguillarum*.

Challenge procedure

To assess whether protection was conferred by passive immunization, fish were challenged with living *V. anguillarum*. For this purpose, *V. anguillarum* cells were grown at 20°C for 24 h in trypticase soy broth supplemented with 1.5% NaCl. The broth culture was then diluted to 1 : 1 000 with saline (0.9% NaCl), and 0.1 ml of the diluted material was administered intramuscularly to test fish held in fresh water at 17°C. This dose contained approximately 2.5×10^5 viable cells and usually killed an unimmunized fish within 72 h.

Following the challenge, fish that became moribund were periodically tested to verify that the injected pathogen was present. Verification was presumptive and was based on reisolating the pathogen and showing it to be sensitive to the vibriostatic compound 0-129 (2,4-diamino-6, 7 di-iso propyl pteridine phosphate).

Antigens

To prepare bacterins and bacterial cells for serological tests, 24-h cultures of strain 700-23 *V. anguillarum* were grown at room temperature in trypticase soy broth with 1.5% NaCl added. The cells were centrifuged at 5 000 × *g* for 30 min and the supernatant decanted. The cells were then washed three times in saline and prepared for use as antigen by either heat or formalin treatment. Washed bacterial cells were heat-killed by placing a 5% suspension of cells in saline in a boiling water bath for 2.5 h; after cooling, 0.5% phenol was added as a preservative. Formalin treatment consisted of incubating equal volumes of washed cells and a saline solution containing 0.6% formalin for one week at room temperature. The suspension was then centrifuged, the supernatant discarded and the bacteria resuspended to a concentration of 5% in saline; finally, 0.5% phenol was added. *E. coli* was heat-killed in the same manner as *V. anguillarum*. All antigens were stored at 8°C.

Immunizations

24 adult rainbow trout were divided into four lots of six fish and each fish was injected intraperitoneally at weekly intervals for 8 weeks with one of the following inocula:

Lot I — 0.5 ml of an emulsion containing equal volumes of a 5% formalin-killed suspension of *V. anguillarum* in saline, and Freund's complete adjuvant.

Lot II — 0.5 ml of a 2.5% formalin-killed suspension of *V. anguillarum* in saline.

Lot III — 0.5 ml of an emulsion containing equal volumes of a 5% heat-killed

suspension of *V. anguillarum* in saline, and Freund's complete adjuvant.

Lot IV — 0.5 ml of a 2.5% heat-killed suspension of *V. anguillarum* in saline.

In addition, two 800-g rainbow trout were each injected with 0.5 ml of a 5% suspension of heat-killed *E. coli* in saline at weekly intervals for 6 weeks.

Serological tests

Sera were tested for agglutinating titers with a microtiter apparatus (Microtiter, Cook Engineering Co.)*. Standard twofold saline dilutions of sera were tested for agglutination activity against the heat-killed *V. anguillarum* antigen. Titers were read after the reaction had proceeded for 1 h at room temperature and overnight at 7°C.

Absorption of antiserum

Immune serum was allowed to react for 30 min in an ice bath with an equal volume of wet-packed cells of heat-killed *V. anguillarum*. Resulting samples were centrifuged. This absorption procedure was repeated until the antiserum no longer agglutinated heat-killed *V. anguillarum*. The protein content (measured as absorbance at 280 nm) of the unabsorbed serum was adjusted to that of the absorbed serum using saline as the diluent.

Passive transfer of serum

The following sera were injected intraperitoneally (0.5 ml/fish) into juvenile rainbow trout (at the same time as the fish were challenged with approx. 2.5×10^5 live *V. anguillarum*):

(1) Rainbow trout anti-*V. anguillarum* serum (pooled) (agglutinating titer 4096).

(2) The same pool (1) absorbed with heat-killed *V. anguillarum* until agglutinating activity was abolished.

(3) Normal rainbow trout serum.

(4) Rainbow trout anti-*E. coli* serum (agglutinating titer 256).

(5) Rabbit anti-*V. anguillarum* serum (agglutinating titer 64).

Passive immunization and rate of antibody appearance

To determine the time required for intraperitoneally injected antibody to become systemic, 2 ml of the anti-*V. anguillarum* serum pool (serum 1) were injected into each of three trout weighing about 75 g each. The fish were bled prior to injection and at intervals after injection (0.1—0.2 ml per bleeding) and the serum harvested and assayed for the presence of agglutinins.

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*Trade names referred to in this publication do not imply endorsement of commercial products.

Duration of passive immunity

110 juvenile rainbow trout (approx. 40 g each) were used to determine the duration of passive protection, and to attempt to relate serum agglutinating titers to protection. 50 fish were given 0.5-ml intraperitoneal injections of normal rainbow trout serum (0 titer) and sixty were given 0.5-ml doses of the immune serum pool (4096 titer). The two groups were kept at 15°C in separate tanks in the holding room.

Seven to ten fish were randomly selected from each group at intervals for challenge experiments. Immediately prior to challenge, blood samples (0.1—0.2 ml) were taken from each fish in the subgroups and serum agglutinin titers for *V. anguillarum* were determined.

RESULTS

Active immunization

Each fish immunized with *V. anguillarum* antigens was bled 62 days after immunization and the agglutinin titer determined. Fish were exsanguinated at 75 days and the serum was frozen. The fish that received the heat-killed *V. anguillarum* in complete Freund's adjuvant produced the highest agglutinin titers of the four antigen preparations used (Table I).

TABLE I

Agglutinin titers developed by trout immunized with *Vibrio anguillarum*

Lot	Antigen	Number of fish	Titer (geometric mean)
I	Formalin-killed with adjuvant	4*	6 208
II	Formalin-killed with saline	6	1 448
III	Heat-killed with adjuvant	6	92 681
IV	Heat-killed with saline	6	2 896

*Two fish in Lot I died before completion of immunization.

Passive immunization and challenge experiments

21 of 22 juvenile rainbow trout, passively immunized with the high titer (4096) trout anti-*V. anguillarum* serum pool and challenged with live *V. anguillarum*, were alive 120 h after challenge and showed no evidence of infection. One of the fish died that received the hyperimmune serum pool, and 20 controls that received 0.5 ml of saline intraperitoneally were dead within 48 h with lesions typical of vibriosis (Fig.1).

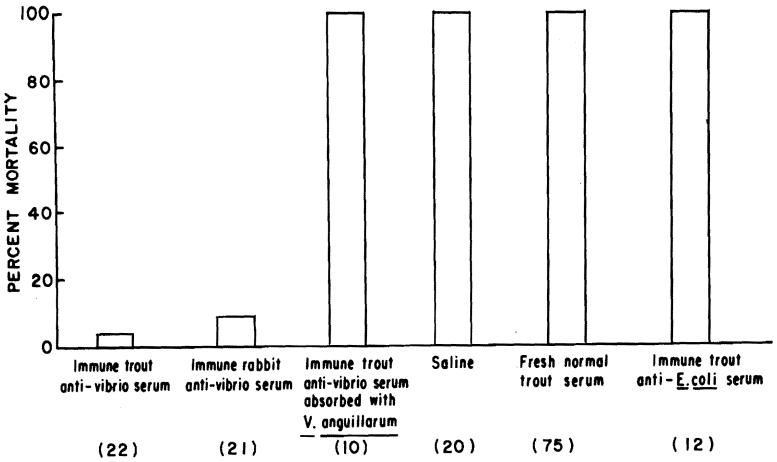

Fig.1. Protection of juvenile trout against vibriosis following passive immunization. Numbers in parentheses are numbers of fish tested.

36 h after challenge with *V. anguillarum* the ten fish given the absorbed serum were dead. The 12 fish that were passively immunized with *E. coli* antiserum and challenged with *V. anguillarum* were dead within 48 h (Fig.1). There were no mortalities in six unchallenged control fish injected with *E. coli* antiserum.

Passive immunization and rate of antibody appearance

1 h after the intraperitoneal injection all three fish contained detectable anti-*V. anguillarum* agglutinins in their sera (Fig.2). (In a separate experiment, anti-*V. anguillarum* agglutinins were detected in serum as early as 10 min after intraperitoneal injection.) The titers in these fish reached a peak (512) 96 h after injection.

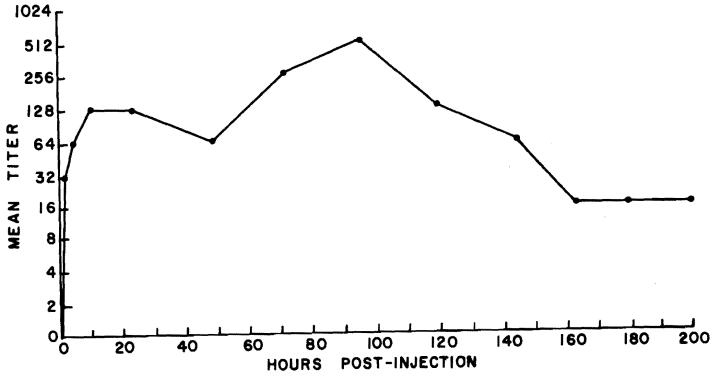

Fig. 2. Levels of agglutinins in the serum of fish at various times following passive immunization by the intraperitoneal route.

Duration of passive immunity

Each passively immunized fish was protected from parenteral *V. anguillarum* challenge for as long as 11 days after the injection of immune trout anti-*V. anguillarum* serum (Fig. 3). The agglutinin titers declined steadily following vaccination and protection against vibriosis began to decrease after day 11; at day 64 there was still some immunity to *V. anguillarum*. Control fish (50) had no detectable agglutinins for the duration of the experiment and all died within 48 h of each challenge.

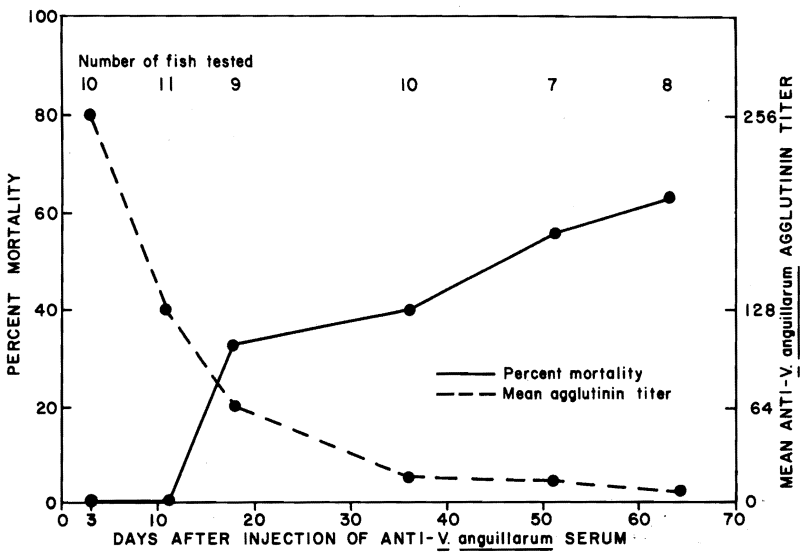

Fig. 3. Relation between passive immunity and circulating antibody levels in trout injected with trout anti-*V. anguillarum* serum and challenged with *V. anguillarum*.

Passive immunization using rabbit antiserum

21 juvenile steelhead, each given 0.5 ml of rabbit anti-*V. anguillarum* serum (titer 64) intraperitoneally, were also challenged at the same time. After 48 h at 17°C, 19 of these fish were still alive. The six controls that received 0.5 ml saline and an equivalent challenge of *V. anguillarum* had died (Fig. 1).

DISCUSSION AND CONCLUSIONS

Our studies have revealed only one protective factor in serum — specific antibody. When anti-*V. anguillarum* agglutinins were removed by absorption with heat-killed *V. anguillarum*, protection against experimental vibriosis was eliminated. Furthermore, anti-*E. coli* serum did not demonstrably protect against vibriosis. These results do not rule out the existence of non-antibody

antibacterial factors in trout serum, but only reflect that none was demonstrated by the methods used. Although the mechanism of antibody-mediated protection was not determined by these experiments, the antibodies could be acting as opsonins and/or they could be fixing complement, bringing about the subsequent lysis of *V. anguillarum*.

It should be noted that the agglutinating titer of the trout anti-*E. coli* serum was much lower (256) than the anti-*V. anguillarum* serum (4096). Nevertheless, trout anti-*V. anguillarum* sera with similar bacterial agglutinating activity as the trout anti-*E. coli* serum used in these experiments have provided demonstrable protection against experimentally induced vibriosis. Therefore, it can be reasonably concluded that the lack of protection from injected anti-*E. coli* serum was due to a lack of specific anti-*V. anguillarum* antibody.

Heat-killed *V. anguillarum* with complete Freund's adjuvant induced the greatest serological response, suggesting that vaccines from heat-killed bacteria would protect fish against vibriosis better than formalin-killed bacterial preparations. Alexander and Soltys (1973) also demonstrated that autoclaved bacterins of *Pasteurella multocida*, compared with formalinized bacterins, stimulated higher titers of agglutinins in turkeys.

Rainbow trout passively immunized with allogeneic serum were afforded effective protection against *V. anguillarum* challenge for a period of 60 days. If a natural challenge in salt water is less potent than this injected challenge, susceptible fish might be protected longer. The experiments indicated a relation between agglutinating titer and protection against experimental challenge with *V. anguillarum*. Whether similar titers are necessary for protection in natural infections or other diseases can only be inferred.

The gonads of hyperimmune female trout were tested for possible transfer of agglutinating antibodies. Although anti-*Vibrio* agglutinins with titers of 1 : 240 were found in the ovarian fluid, none was found in the eggs.

Rabbit or trout antiserum made against *V. anguillarum* appears useful for passive protection of particularly valuable fish during those months when vibriosis is prevalent in the Pacific Northwest. In veterinary medicine large quantities of immune serum are used therapeutically in certain infectious disease as an adjunct to chemotherapy. The early (10 min) appearance of serum antibody after intraperitoneal injection suggests that passive transfer of antibody to diseased fish may also be of therapeutic value.

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