Development of passive immunotherapy for control of infectious hematopoietic necrosis

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ABSTRACT· When rainbow trout *Oncorhynchus mykiss* (mean weight, 4.3 g) were injected with 100 µl of anti-infectious hematopoietic necrosis virus (IHNV) rainbow trout immune serum (IHNV neutralizing titer 640) immediately following waterborne exposure to 10^5 plaque-forming units ml^{-1} of IHNV, relative protection was 91 % compared to fish injected with normal serum (titer < 20). Fish injected with immune serum at 24 and 48 h post-exposure to virus exhibited relative protection of 88 and 75 %, respectively. Less protection was observed in fish injected 3 or 4 d post-exposure. Endogenously produced virus neutralizing titers in surviving fish injected at 0 and 24 h post-exposure were not detected but fish injected at 48 h produced a significant humoral response. Juvenile rainbow trout (mean weight 1 g) receiving injections of 50 µl of serum having a low plaque neutralization titer of 20 to 40 resulted in relative protection of 67 to 82 % compared to fish injected with saline. When the same volume of a higher-titered serum was injected, greater protection was generally observed. These results suggested that low neutralization titers could be sufficient for significant protection against IHN disease. In 1 field trial, mortality due to IHN was lower in the group that received the passive immunotherapy compared to a group that was left untreated.

KEY WORDS: Rhabdovirus · Rainbow trout · Passive immunization

INTRODUCTION

Strategies currently used to prevent and minimize economic impact due to disease in the intensive culture of rainbow trout *Oncorhynchus mykiss* include prudent fish health management (e.g. pathogen-free water supplies, bird exclusion devices), vaccination (induction of active immunity), and treatment with therapeutants. Passive immunity examined experimentally by injection of sera from immune trout to naive trout has been found to provide protection from fish pathogens including *Vibrio anguillarum* (Harrell et al. 1975), *Yersinia ruckeri* (Oleson 1991), and infectious hematopoietic necrosis virus (IHNV; Amend & Smith 1974). Passive serum transfer has been examined as a prophylactic and/or therapeutic strategy in the field for the control of furunculosis in 3 salmonid species (Turgeon & Elazhary 1992). Although injection was required and duration of protection was limited, this strategy could be useful on valuable or small fish populations. Additionally, passive immunization studies can provide important information about immunogenic proteins, requirement for multiple vaccine strains, pathogenesis of microorganisms, and titer of antibody required for protection. We previously reported on protection against IHNV after passive transfer of anti-IHNV immune serum (LaPatra et al. 1993). When rainbow trout (mean weight, 1 g) were injected with 50 to 200 µl of pooled serum from juveniles (IHNV neutralizing titer 1280) or adults (titer 640) that had experienced a natural infection with IHNV and had survived, relative protection was 63 to 100 % against IHNV compared to rainbow trout injected with normal serum (titer < 20) or saline. The objectives of this study were to determine (1) if injection of fish serum having IHNV neutralizing activity effectively reduced mortality at different times post-exposure to IHNV, (2) if fish that were protected by passively transferred antibodies still responded humorally, (3) the minimum neutralization titer of serum required for protection after passive transfer, and (4) if passive immunotherapy could be used as an IHN management strategy.

MATERIALS AND METHODS

Passive immunotherapy and virus challenges. Virus challenges and passive immunizations were done using procedures previously described for laboratory testing of passive immunity (LaPatra et al. 1991, 1993). Briefly, standardized waterborne exposures to IHNV [10^5 plaque-forming units (pfu) ml^{-1}] were done in an amount of water equaling 10 times the total weight (g) of the fish for 1 h in closed systems with aeration. For passive immunization, fish were anesthetized and injected intraperitoneally (ip) with rainbow trout immune serum (IHNV neutralization titer ≥ 20), normal serum (titer, <20), or saline. A group of 1500 Clear Springs stock rainbow trout (mean weight 4.3 g) was waterborne-exposed to 10^5 pfu ml^{-1} of IHNV isolate 220-90, and 300 additional fish from the same population were handled similarly but not exposed to the virus. Immediately post-exposure (time 0) and at 24 h intervals for 4 d, 100 fish were removed from the IHNV-infected group and 50 of these were injected ip with 100 µl of rainbow trout immune serum (IHNV neutralizing titer 640) or normal serum. Each 50 fish treatment group at each test interval was split into duplicate 25 fish subgroups in separate 19 l aquaria supplied with 15 °C spring water treated by ultraviolet light. Additionally, 25 sham-infected fish were injected with immune or normal serum immediately post-exposure and at 48 h intervals for 4 d and placed in separate aquaria. Mean cumulative percent mortality and relative percent protection were calculated for each group. Cumulative percent mortality of the replicates was analyzed by analysis of variance (ANOVA) on transformed (arcsin $\sqrt{\text{percentage}}$) data (Snedecor & Cochran 1967) and pairwise comparisons were done with the Mann-Whitney Rank Sum Tests using the Sigma Stat statistical software (Jandel Scientific Co.).

Control and challenged fish from each of the treatment groups were bled 28 d after exposure to virus for determination of serum neutralization titers using a complement dependent 50 % plaque neutralization titer (PNT) assay (LaPatra et al. 1993, Ristow et al. 1993). Briefly, blood was obtained from fish that had been lethally anesthetized, either by caudal puncture or by caudal severance. Blood samples were placed at 4 °C, allowed to clot, and centrifuged for 10 min at $1600 \times g$. Serum samples were heat-inactivated at 45 °C for 30 min and a 2-fold dilution series was made. An equal volume of diluted IHNV (2000 pfu ml^{-1}) and complement (1 10 dilution), obtained from disease-free rainbow trout that had not been fed for at least 2 wk, was added to each serum dilution. Samples were plaque assayed in duplicate for each serum dilution tested on epithelioma papulosum cyprini (EPC) monolayers. Anti-IHNV neutralization titers were reported as the reciprocal of the highest serum dilution that resulted in a 50 % reduction in the average number of plaques detected in negative controls. Significant differences among treatments were analyzed by a chi-square test.

Minimum neutralization titer for protection. Two studies were done to determine the minimum neutralization titer of trout serum required for protection in juvenile rainbow trout (mean weight 1 g) after passive transfer. In the first study, 50 rainbow trout were injected with 50 µl of trout serum having a 50 % PNT of 1280, 320, 80, or 20. In the second study 50 fish were injected with 50 µl of trout serum that had a 50 % PNT of 640, 160, 40, or 10. In each test, the lower antibody concentrations were made by diluting serum having a starting titer of 1280 or 640 with phosphate buffered saline (PBS) in 4-fold increments. One group of fish in each test was injected with PBS only. Duplicate 25 fish groups from each treatment were challenged with 10^5 pfu ml^{-1} of IHNV by standard procedures. Control fish were injected with 50 µl of undiluted immune serum and handled similarly but not exposed to virus. Average cumulative percent mortality and mean number of days to death were calculated for each group. Cumulative percent mortality of the replicates was analyzed by ANOVA on transformed data and pairwise comparisons were done using the Mann-Whitney Rank Sum Tests. Relative percent protection (RPP) for groups injected with immune serum was calculated by the following formula:

$$\text{RPP} = 1 - \frac{(\text{cumulative \% mortality in fish injected with immune serum})}{(\text{cumulative \% mortality in fish injected with normal serum})}$$

Passively immunized groups for all tests were held in separate 19 l aquaria, and monitored for mortalities for 28 d. Each day, at least 20 % of the fish that died on that day were examined for virus. Virus concentrations of whole fish or kidney-spleen-liver homogenates were determined. Quantitation of virus used in fish exposures or isolated from dead fish was accomplished by plaque assay procedures (LaPatra et al. 1989).

Passive immunization field tests. A group of approximately 2000 fish (mean weight 64 g) were diagnosed with spontaneous infectious hematopoietic necrosis (IHN) after a slight increase in mortality. Half of this group (1000 fish) were each injected ip with 1 ml of serum containing IHNV-neutralizing antibodies (titer 640) to determine the effectiveness of passive immunotherapy in the field. Fish were monitored for mortality and 15 from each treatment group were nonlethally bled every 2 wk for 10 wk beginning 14 d post-injection to determine IHNV neutralization titers.

RESULTS

Passive immunization after exposure to IHNV

Fish injected with immune serum immediately after exposure to IHNV exhibited relative protection of 91% compared to fish injected with normal serum. Fish injected at 24 and 48 h post-exposure exhibited relative protection of 88 and 75%, respectively, compared to controls. Less protection was observed in fish injected at the later time intervals (Table 1). There was not a significant difference (p > 0.5) among all mean cumulative percent mortality values for fish challenged with IHNV and either injected with immune or normal serum. However, there was a significant difference (p = 0.02) in the mean values between the different treatments over all days post-infection. Virus was detected in 87% (111/127) of the dead rainbow trout examined. Virus concentrations determined on 50 of these fish ranged from 10^2 to $>10^{7.3}$ pfu g^{-1} with a mean titer of 10^6 pfu g^{-1}

IHNV neutralization activity in fish serum

Fish injected with immune serum at 0 and 24 h post-exposure to IHNV exhibited low mortality, and antibody concentrations in surviving fish were significantly different (p < 0.03) than in IHN-survivors injected with normal serum. However, fish injected 48 h post-exposure produced a strong humoral response and were also effectively protected (Table 2). Antibody titers detected in these fish were not significantly different (p > 0.05) from those of IHN-survivors injected with normal serum. Control fish not challenged with IHNV but injected with immune serum still had a low level of neutralizing activity (≥ 20) in the serum after the experimental period whereas control fish injected with normal serum had no detectable anti-IHNV activity. Additionally, survivors that were injected

with immune serum just post-exposure to IHNV (time 0) had more antibody-negative fish than control fish injected with immune serum but not exposed to IHNV.

Passive immunotherapy field trial

The results of 1 field trial indicated that approximately 44% fewer fish were lost in the immune serum injected group (43/1000) compared to the group that was not injected (77/1000). Non-lethal blood samples obtained at 2 wk intervals showed that passively immunized rainbow trout had higher prevalences of anti-IHNV positive fish and higher mean antibody titers at 2 and 4 wk post-injection. The control group generally had higher mean titers and prevalences toward the end of the sampling period (6, 8, and 10 wk) (Table 3).

Passive immunization threshold level of protection

Two experiments indicated that injection of 50 µl of serum with a PNT of 20 to 40 into 1 g rainbow trout provided relative protection of 67 to 82%. If the same volume of higher-titered serum was delivered, greater protection was generally observed (Table 4). There was a highly significant difference (p = 0.003) in the first test in the mean cumulative percent mortality values for fish injected with immune serum compared to fish injected with saline. There was not a significant difference (p > 0.5) in the mean values for fish injected with immune serum. In the second test there was also a highly significant difference (p = 0.0001) in cumulative percent mortality for fish injected with immune serum compared to fish injected with saline. However, there was also a significant difference (p < 0.05) in mortality for fish injected with immune serum diluted 1:64 (estimated titer 10) compared to other immune serum injected groups. Virus was detected in 84%

Table 1. *Oncorhynchus mykiss*. Cumulative mortalities in young rainbow trout challenged with infectious hematopoietic necrosis virus (IHNV) after passive transfer of immune serum beginning immediately after exposure to IHNV (time 0) and at 24 h intervals for 4 d. Trout either waterborne-exposed to 10^5 pfu ml^{-1} IHNV or sham-exposed, were injected with normal serum (titer < 20) or immune serum (titer 640) that contained anti-IHNV antibodies. Relative protection: % of fish injected with immune serum compared to fish injected with normal serum and challenged with IHNV; nd = not done

| Time post-infection (d) | Cumulative mortality (%) | | | | Relative protection (%) |
| | IHNV-exposed | | Sham-exposed | | |
	Immune	Normal	Immune	Normal	
0	6 (3/49)	69 (35/51)	4 (1/24)	4 (1/24)	91
1	8 (4/50)	69 (34/49)	nd	nd	88
2	18 (9/50)	72 (36/50)	0 (0/24)	0 (0/25)	75
3	42 (21/50)	60 (30/50)	nd	nd	30
4	50 (25/50)	70 (35/50)	0 (0/25)	8 (2/25)	29

Table 2. *Oncorhynchus mykiss*. IHNV antibody titers detected 28 d post-exposure in IHNV-exposed and sham-treated groups. Fish (mean weight 4.3 g) were injected with 100 μl of immune serum (IHNV neutralizing titer 640) or with normal serum. Results given as the highest serum titer resulting in a 50% reduction in the number of plaques detected in negative serum controls. ns: no sample

Fish no.		IHNV-exposed					Sham-exposed		
Day injected:	0	1	2	3	4		0	2	4
Fish injected with immune serum									
1	40	20	160	320	80		< 20	20	< 20
2	20	20	320	80	80		20	20	80
3	< 20	80	40	160	160		20	20	80
4	20	20	≥ 640	160	160		80	80	ns
5	< 20	< 20	320	320	320		40	< 20	ns
6	< 20	80	160	ns	ns		ns	ns	ns
7	20	20	40	ns	ns		ns	ns	ns
8	< 20	20	160	ns	ns		ns	ns	ns
Fish injected with normal serum									
1	160	80	80	160	≥ 640		< 20	< 20	< 20
2	80	160	160	160	320		< 20	< 20	< 20
3	160	≥ 640	≥ 640	160	160		< 20	< 20	< 20
4	ns	ns	ns	≥ 640	ns		ns	ns	ns

(43/51) and 92% (61/66) of the dead rainbow trout examined in each experiment. Virus concentrations determined on 75 of these fish ranged from 10^2 to $> 10^{7.3}$ pfu g^{-1} with a mean titer of $10^{6.4}$ pfu g^{-1}.

DISCUSSION

The passive transfer of anti-IHNV serum by injection into fish 0 to 48 h after exposure to high concentrations of IHNV provided relative protection of 75 to 91% compared to fish injected with normal serum. Less protection was observed in fish injected at the later time points. Water temperature has been shown to effect mean number of days (MDD) to death in rainbow trout waterborne exposed to IHNV and may influence the efficacy of passive immunization. Hetrick et al. (1979) reported the MDD was only 7 d at 18°C compared to 17 d at 3°C. These tests were conducted at water temperatures of 15°C which is considered the optimum for replication of IHNV (Wolf 1988). The mean

days-to-death in IHNV exposed groups injected with normal serum averaged 7.6 d (data not shown) but good protection was only observed up to 48 h post-exposure to IHNV. Perhaps the virus had induced irreversible tissue damage after 48 h such that there could be no recovery of fish regardless of passive serum transfer. Tests of passive serum transfer with salmonids reared at colder temperatures may be more effective over longer durations because viral pathogenesis would be slowed. The half-life of the exogenously supplied immunoglobulin may also be extended because of decreased metabolic activity of fish in cooler water. The salmonid species tested as well as size and age may also affect results because these have been shown to impact IHNV-susceptibility (LaPatra et al. 1990). Additionally, dose of viral challenge and susceptibility differences within an experimental group are important parameters to consider when interpreting results for development of passive immunotherapy strategies.

Passively transferred immune serum provided immediate protection against IHN disease, but for longer

Table 3. *Oncorhynchus mykiss*. Neutralizing antibody titers to IHNV detected in rainbow trout surviving a natural infection of IHNV as part of a field evaluation for the passive immunization strategy. PNT: plaque neutralization titer

Time post-injection (wk)	Immune serum injected			Non-injected		
	Proportion PNT-positive	PNT mean	PNT range	Proportion PNT-positive	PNT mean	PNT range
2	8/15	19	< 20–80	3/15	5	< 20–40
4	10/15	44	< 20–320	8/15	23	< 20–80
6	11/15	43	< 20–160	13/15	91	< 20–320
8	3/15	43	< 20–320	7/15	16	< 20–80
10	1/15	3	< 20–40	10/15	31	< 20–160

Table 4. *Oncorhynchus mykiss*. Passive immunization of rainbow trout (mean weight 1 g) with different concentrations of trout anti-IHNV immune serum. A titer of 0 indicates injection with 50 µl phosphate-buffered saline; control fish were injected with 50 µl of serum with a neutralization titer of 640 and handled equivalently but not exposed to virus. nm: no mortality

Anti-IHNV neutralization titer	Average cumulative mortality (%)	Mean days-to-death	Relative protection (%)
Test 1			
1280	4 (2/46)	9	96
320	2 (1/49)	23	98
80	15 (7/46)	19	84
20	32 (16/50)	17	67
0	96 (47/49)	9	
Control	0 (0/20)	nm	
Test 2			
640	21 (10/48)	15	79
160	26 (12/47)	16	74
40	18 (9/50)	16	82
10	57 (26/46)	14	43
0	100 (50/50)	8	
Control	0 (0/20)	nm	

duration of protection a specific humoral response would be required. Fish injected with IHNV-immune serum at 0 and 24 h post-exposure to IHNV exhibited low mortality and negligible antibody concentrations in surviving fish, whereas, fish injected 48 h post-exposure produced a significant humoral response and were also effectively protected. Additionally, survivors of IHN that were injected with immune serum immediately after exposure to IHNV (time 0) had more antibody-negative fish than control fish injected with immune serum but not exposed to IHNV. A possible explanation is that challenged fish were depleted of exogenous antibody through binding with specific antigen during the defense against an IHNV-infection. Passively transferred antibody minimizes disease by neutralizing virus infecting the host but it may also inhibit a specific immune response by reducing antigen concentration below the threshold required for humoral stimulation. Ellis et al. (1992) reported that exposure of vaccinated fish to high and persistent *Aeromonas salmonicida* levels accelerated the decrease in protective antibody levels. Some antibody may also be degraded and lost due to normal metabolic processes. Hence, after the 28 d monitoring period, minimal passively transferred antibody was detected in immune serum injected mock-infected controls.

Passive immunotherapy could be used as an emergency management strategy if an important group of fish were diagnosed with IHN in the early stages of an epizootic. The results of 1 field trial indicated that approximately 44 % fewer fish were lost in the immune serum injected group compared to the group that was not injected. However, in both cases the mortality was low. The fish were large (64 g) when mortality due to IHN was detected and were divided into 2 separate raceways. Reducing fish densities possibly helped moderate the severity of the epizootic in both groups. Handling stresses associated with injection may also have exacerbated the disease. However, the passively immunized group that was injected and moved to a different raceway still had less mortality than the unhandled controls. Similar trends of antibody titers and prevalence of antibody-positive fish detected in the passively immunized group were also observed in experimental groups injected at different times after waterborne exposure to IHNV. Passively transferred antibodies were used in defense of an IHNV infection and reduced a specific immune response because antigen concentration was kept low. The results from the untreated fish are also in agreement with previous studies that examined the antibody kinetics in unimmunized fish exposed to IHNV. Low titers and prevalences were detected at 2 wk but all fish responded with high titers by 6 wk (LaPatra et al. 1993).

The minimum IHNV neutralizing titer required to achieve protection against clinical disease has been unclear. This information is important for the development of effective passive immunization therapies. Our 2 experiments indicated that injection of 50 µl of serum with a PNT of 20 to 40 into 1 g rainbow trout provided relative protection of 67 to 82 %. If the same volume of a higher-titered serum was delivered, greater protection was generally observed. These results suggest that low IHNV neutralizing activity, whether obtained by passive immunization or by other strategies, could provide protection or minimize the severity of an IHN epizootic. Antibody titers reported for IHN survivors are highly variable (Hattenberger-Baudouy et al. 1989, Jørgensen et al. 1991, Ristow et al. 1993). However, in naturally infected populations, protection against reinfection is generally observed (Busch 1983) suggesting that low antibody titers are sufficient for strong protection. Although decreased susceptibility to IHNV has been reported with increasing fish size and age (LaPatra et al. 1990) IHN epizootics have been reported in large (100 to 500 g) rainbow trout (Busch 1983) and Atlantic salmon *Salmo salar* (Armstrong et al. 1993). Because mortality in large fish can have severe economic consequences, passive immunotherapy may be a potential strategy for use on valuable fish. Our results suggest that this type of strategy may have the immediate benefit of reduced mortality and

may also allow for endogenous humoral immunity. Monitoring of IHNV neutralizing antibody concentrations could also be used as a predictor of the IHNV susceptibility of a group of fish and the effectiveness of IHN control strategies. Future studies should investigate the practicality of producing large volumes of anti-IHNV antiserum that would be required for widespread use of passive immunotherapy and the use of purified immunoglobulin rather than serum for prevention of sensitization and/or serum sickness if reinjections are required.

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