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Induction of protection from infectious hematopoietic necrosis virus in rainbow trout *Oncorhynchus mykiss* by pre-exposure to the avirulent cutthroat trout virus (CTV)

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ABSTRACT: Juvenile rainbow trout *Oncorhynchus mykiss* exposed to waterborne live cutthroat trout virus (CTV) showed increased resistance to experimental challenge with infectious hematopoietic necrosis virus (IHNV). Pre-exposure to CTV caused a relative percent survival (RPS) of 70 when compared to CTV-mock-treated groups challenged with IHNV. Additionally, the mean day to death was 10 d for CTV-exposed versus 8 d for the CTV-mock-treated group. Protection was obtained following exposures as brief as 5 min but was greatest among trout exposed for 1 h to CTV and then challenged 1 wk later with IHNV. Protection was observed for up to 4 wk following CTV exposures but absent at 6 wk. Concentrations of serum anti-IHNV neutralizing antibodies were significantly higher ($p = 0.007$) among trout previously exposed to CTV when compared to the mock-treated group 5 wk following challenge with IHNV. Both groups (CTV and CTV-mock) surviving the first IHNV exposure were solidly protected to a second IHNV challenge. The mechanisms for the viral mediated resistance induced by CTV is unknown, but the virus was shown to be a potent inducer of interferon-like activity in anterior kidney cells isolated from rainbow trout.

KEY WORDS: Fish virus · Interferon · Viral interference · IHNV · CTV

INTRODUCTION

Co-infection with 2 viruses has been reported among salmonid fish but the potential interactions between these agents and the host's immune response are poorly understood. This is in part due to a general lack of knowledge regarding viral pathogenesis in fish and in particular the mechanisms underlying natural and acquired immunity to these agents. Mulcahy & Fryer (1976) and LaPatra et al. (1993a) reported co-infections with infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) among individual juvenile rainbow trout *Oncorhynchus mykiss* in the states of Oregon and Idaho, USA, respectively. Similarly, de Kinkelin et al. (1992) showed that rainbow trout could be infected with both IPNV and viral hemorrhagic septicemia virus (VSHV). *In vitro* studies have also demonstrated the ability of the rainbow trout gonad (RTG-2) cell line to support co-infections with IPNV and VHSV (Wolf & Vestergård-Jørgensen 1970).

The first information on the potential interaction of these agents in the host was reported by de Kinkelin et al. (1992). They were able to show in co-infections with IPNV and VSHV that young rainbow trout infected with IPNV and later exposed to VHSV showed signifi-

cantly greater survival than parallel groups of trout not previously infected with IPNV. The basis for the virus-mediated interference was suspected to be due to interferon (IFN) but none was detected at the levels of sensitivity for the IFN assay in groups of trout prior to challenge with VHSV. The ability of trout alevins to produce IFN in response to IPNV infections, however, has been reported (Dorson et al. 1992).

In several trials in 2 different laboratories reported here, we demonstrate that infections with the cutthroat trout virus (CTV), an avirulent salmonid virus (Hedrick et al. 1991), can create a resistant state among rainbow trout alevins to subsequent infections with IHNV. Furthermore, we demonstrate the longevity of this response and the resultant effect of prior CTV infections on the development of serum neutralizing antibodies and immunity to IHNV. Lastly, we demonstrate that CTV has the potential to induce the production of antiviral activity following inoculation of isolated anterior kidney leucocytes from juvenile rainbow trout.

MATERIALS AND METHODS

Viruses. CTV was originally isolated in 1989 from healthy adult cutthroat trout *Oncorhynchus clarki* by Yun & Hedrick (1988) and its properties, including the ability to infect additional hosts, are described in more detail by Hedrick et al. (1991). The 039-82 and the 220-90 strains of IHNV were isolated from rainbow trout *O. mykiss* in the Hagerman Valley, Idaho (LaPatra et al. 1991). Experiments conducted at the University of California, Davis, California, USA (Trial 1) utilized IHNV strain 039-82 while those at Clear Springs Food Co., Buhl, Idaho (Trials 2 and 3) employed IHNV strain 220-90, a more virulent and more recently obtained isolate.

Cell lines, virus growth and titration. The CTV was grown in CHSE-214 (Lannan et al. 1984) cells (ATCC CRL 1681) and IHNV in the *epithelioma papillosum cyprini* (EPC) line (Fijan et al. 1983). Both cell lines were maintained in minimal essential medium (MEM) with Earle's salts supplemented with 10% fetal bovine serum (FBS; HyClone Lab., Inc., Logan, UT, USA), 50 I.U. ml^{-1} penicillin, 50 µg ml^{-1} streptomycin and 2 mM L-glutamine. Cells were propagated in either 150 cm^2 flasks or 48- or 24-well dishes at 20 to 25°C. For virus propagation, cells were infected at multiplicities of infection (MOI) of 0.001 to 5 pfu $cell^{-1}$. After adsorption for 30 to 60 min at 18 to 25°C, 20 ml of MEM with 2% FBS were added to 150 cm^2 flasks containing EPC or CHSE-214 cells which were then incubated at 15 to 20°C until cytopathic effects (CPE) were complete (approximately 8 to 14 d). Viruses were titrated by $TCID_{50}$ (50% tissue culture infective dose) analyses (CTV) or by plaque titration (IHNV) on their respective cell lines as previously described (Hedrick et al. 1991, LaPatra et al. 1993b). The level of neutralizing antibodies present in the serum of trout was detected by methods described by Hattenberger-Baudouy et al. (1989) as modified by LaPatra et al. (1993b).

Fish challenges. The effects of pre-exposure to the avirulent CTV on subsequent challenges with IHNV were examined in 3 separate trials with juvenile rainbow trout. The fish in the 3 trials were obtained from stocks with no history of IHNV or CTV. The broodstocks from which juveniles were obtained have been examined each of the last 3 years without any evidence of either viral agent.

In the first trial, 520 rainbow trout (2.1 g) were separated into 2 equal groups in 130 l aquaria and 1 was exposed to $10^{6.5}$ $TCID_{50}$ ml^{-1} of CTV in 2.5 l of water at 15°C for 3 h (CTV exposed). The other group was exposed under the same conditions to a dilution of culture supernatant from uninfected CHSE-214 cells equal to that used for the CTV-exposed group (mock treated). After 2 wk at a water temperature of 15°C, 5 fish from each group were assayed for the presence of virus by inoculation of CHSE-214 cells and 160 fish (now 2.8 g) from each group were then separated into 2 subgroups of 80 fish. One of the subgroups from each of the CTV-exposed and the mock-treated groups was challenged with the 039-82 strain of IHNV at $10^{7.4}$ $TCID_{50}$ ml^{-1} for 1 h before resuming the flow of 15°C well water to the aquaria. The 2 remaining groups were treated in the same manner but were exposed only to culture medium from uninfected cells. Dead fish were collected daily for a period of 21 d and the entire viscera and kidney were examined for presence of IHNV by isolation attempts on EPC cells as described by LaPatra et al. (1991). The presence of CTV in the CTV-exposed group at the time of IHNV challenge was examined by isolation attempts from 5 fish. The relative percent survival (RPS) for CTV-exposed and then IHNV-challenged groups was calculated by the following formula:

$$1 - \left(\frac{\text{Cumulative \% mortality CTV-exposed group}}{\text{Cumulative \% mortality mock-treated group}} \right) \times 100$$

The statistical differences between groups were determined by paired *t*-tests at $p < 0.05$.

In the second trial with IHNV, trout (1.3 g) were divided into treatment groups of 500 fish each. One group was exposed to CTV for 5 min, the second to CTV for 1 h, and the third to supernatant from uninfected CHSE-214 cells. All CTV exposures were done at a concentration of 10^5 $TCID_{50}$ ml^{-1}. The fish from each group were then separated so that 20 l tanks receiving 15°C well water each contained 25 fish. At periods of 1, 2, 4 and 6 wk post exposure to CTV,

3 replicate tanks from each original treatment group (except the untreated controls) were challenged with strain 220-90 IHNV at 10^4 pfu ml^{-1} for 1 h. A second series of replicates from each of these treatment groups and the untreated controls were exposed only to supernatant from EPC cells. Virus isolations from a subset of the dead fish were used to confirm the cause of mortality for the 28 d period following challenge with IHNV (LaPatra et al. 1991). The anti-IHNV neutralizing antibody titers found in 5 pooled serum samples obtained from surviving trout from each treatment group were examined at 4 to 8 wk post IHNV challenge.

In the third trial, rainbow trout alevins (2.6 g) were divided into 5 replicate groups of 300 fish each. Two groups were infected with CTV by immersion in 10^5 $TCID_{50}$ ml^{-1} of virus for 1 h at 15 °C. The other 3 groups were exposed to an equal volume of medium from uninfected CHSE-214 cells (mock CTV). The fish were held in 130 l aquaria receiving flow-through water at 15 °C. After 1 wk, 1 CTV and 2 mock-CTV replicate groups were challenged with 10^4 pfu ml^{-1} of IHNV strain 220-90 as previously described (Trial 2). The 2 remaining groups (1 CTV infected and 1 mock CTV) were exposed only to medium and were held under the same conditions. All groups of fish were then held in 15 °C water for 5 wk and fish were confirmed to be infected by isolation of IHNV from a subset of the fish dying during the study.

Fish remaining from Trial 3 in each treatment group (both mock-CTV replicates challenged with IHNV were combined) were examined after 5 wk for the presence of serum antibodies to IHNV as previously described. A total of 22 two-fish pools from each of the IHNV challenged groups (CTV mock, CTV infected) and 11 two-fish pools from controls not exposed to IHNV were examined.

To determine the level of immunity to re-infection in different treatment groups, 75 fish were removed from each tank of survivors or previously unchallenged groups and their average weight determined prior to dividing them into 4 replicate groups in 20 l aquaria. Three replicates from each treatment group were then challenged with 10^5 pfu ml^{-1} of IHNV strain 220-90 as previously described but at a higher dose since the fish were greater in size and more resistant to the virus. The fourth replicate for each treatment was left unchallenged. A representative number of mortalities were examined for IHNV and the experiment was terminated 3 wk after the challenge.

Detection of interferon-like activity. Virus-free juvenile rainbow trout (9 to 13 g) were held in 200 l circular aquaria supplied with fish pathogen-free well water at 11 to 15 °C at the University of Idaho laboratory. The trout were acclimated for 3 wk prior to use in the experiments. All fish were fed a commercial semi-moist pellet ration at the manufacturer's recommended level.

The ability of CTV to stimulate production of antiviral cytokine or interferon-like activity in anterior kidney cells of 7 individual juvenile rainbow trout was examined *in vitro* by a modification of the method described by Renault et al. (1991). Briefly, suspensions of anterior kidney (AK) cells were prepared by removing the anterior one-third of the kidney and pressing it through a sterile stainless steel screen (100 mesh) into cold Hanks' balanced salt solution without calcium or magnesium. Erythrocytes were removed by centrifugation of the cell suspension over Histopaque 1077 (Sigma, St. Louis, MO, USA). The leucocytes were recovered, enumerated by hemocytometer counts and adjusted to 4×10^6 cells ml^{-1} in L-15 medium. In 1 experiment, dilutions of stock virus were added to equal volumes of AK cell suspensions, resulting in final cell densities of 2×10^6 cells ml^{-1} and CTV concentrations of 3, 1.5 or 0.75×10^6 $TCID_{50}$ ml^{-1} and for comparison IHNV strain 220-90 at 5.1×10^6 pfu ml^{-1} in L-15 medium. After 48 h at 16 °C a portion of the supernatants from each culture was harvested from virus-stimulated AK cell cultures by centrifugation and diluted in 2-fold steps from 1:8 to 1:128 with RPMI 1630 medium containing 4 % FBS.

In a second experiment, AK cell cultures from 5 fish were divided into 2 portions and stimulated by CTV at a concentration of 6×10^6 $TCID_{50}$ ml^{-1}. Culture medium was harvested at either 24 or 72 h. Supernatants from IHNV-stimulated cultures were treated with 4 N HCl to inactivate any infectious IHNV: the pH was reduced to 2 and restored to neutrality after 1 h by the addition of 4 N NaOH. The diluted supernatants were transferred to 96-well plates (0.1 ml per well, replicated in 6 wells) containing fresh monolayers of CHSE-214 cells. At the completion of the CHSE-214 cell treatment period (16 h at 16 °C), the medium was removed and the cells challenged with 5000 pfu of strain 220-90 IHNV per well. In addition, 12 other wells were treated but not challenged, providing a subsequent measure of cell density in the absence of any viral cytopathic effect (maximum cell density). Other wells were challenged but not treated, providing an estimate of the cell density resulting from cytolysis of unprotected cells (minimum cell density).

After 72 h incubation (16 °C), the medium was removed and the cell monolayers were fixed and stained with 1 % crystal violet in 20 % ethanol for 30 min. The plates were then air dried and the extent of virus-induced cytolysis quantified by reading the optical density of the stained monolayers with an ELISA reader at a wave length of 600 nm. The mean percent protection (%Px) provided by treatment of the

Table 1 *Oncorhynchus mykiss*. Susceptibility of rainbow trout to infectious hematopoietic necrosis virus (IHNV) following exposures to the cutthroat trout virus (CTV). Rainbow trout (2.1 g) were treated with either culture media from uninfected cells (mock) or cutthroat trout virus for 3 h. At 2 wk post-treatment, 2 groups of ca 80 fish from each treatment group were challenged with IHNV strain 039-82 at a dose of approximately $10^{7.4}$ $TCID_{50}$ ml^{-1} for 1 h before resuming the flow of 15 °C water into the aquaria

Weeks post-treatment	Treatment						
	Mock		3 h CTV			Control (CTV)[a]	
	CPM[b]	MDD[c]	CPM	MDD	RPS[d]	CPM	MDD
2	84	8	25	10	70	4	15

[a]No mortalities occurred in the non-CTV-exposed non-IHNV-challenged group
[b]CPM = cumulative percent mortality
[c]MDD = mean day to death
[d]RPS = relative percent survival when compared to mock-treated group

cells with each dilution (x) of AK cell supernatant was estimated as:

$$\text{Protection (\%Px)} = \frac{x\text{OD(treated, challenged)} - x\text{OD(minimum density)}}{x\text{OD(maximum density)} - x\text{OD(minimum density)}} \times 100$$

The data obtained were plotted against reciprocals of the corresponding supernatant dilutions on log-probit paper, and the dilution providing 50% protection was estimated by interpolation. Antiviral activity was estimated in 50% tissue culture protective dose ($TCPD_{50}$) units ml^{-1}:

$$TCPD_{50}\ U\ ml^{-1} = \frac{1.0\ ml \times (\text{reciprocal of dilution providing 50\% protection})}{0.1\ ml}$$

Statistical analyses. Significance differences among treatments with replicates (Trials 2 and 3) were evaluated by ANOVA or Mann-Whitney Rank Sum Tests using Sigma Stat statistical software (Jandel Scientific Co., San Rafael, CA, USA). Significant differences between means in the concentration of interferon-like activity were determined using Kruskal-Wallis 1-way ANOVA on ranks. All pairwise comparisons were made by Dunn's method (Jandel Scientific Co.).

RESULTS

Trial 1

Rainbow trout previously exposed to CTV showed significantly less ($p < 0.05$) mortality than mock-treated fish when challenged with IHNV; the cumulative mortalities in the 2 groups were 25% and 84%, respectively (Table 1). All dying fish in the IHNV-exposed groups exhibited external signs of IHNV infection and the virus was recovered from all dead fish tested. The mean day to death due to IHNV infection was greater for the CTV-exposed group (10 d) compared to that in the mock-treated group (8 d). Two fish from the CTV-exposed group not challenged with IHNV died during the test but these fish had no signs of disease nor was CTV or IHNV isolated. A sample of 5 fish from each of

Table 2. *Oncorhynchus mykiss*. Susceptibility of rainbow trout to infectious hematopoietic necrosis virus (IHNV) following exposures to the cutthroat trout virus (CTV) for 5 min or 1 h over a period of 6 wk. Rainbow trout were treated with either culture media from uninfected cells (mock) or cutthroat trout virus for 5 min or 1 h. At 1, 2, 4 and 6 wk post-treatment, 3 groups of ca 25 fish from each treatment group were challenged with IHNV strain 220-90 at a dose of approximately 10^4 pfu ml^{-1} for 1 h before resuming the flow of 15 °C water into the aquaria

Weeks	Fish wt (g)	Treatment								
		Mock		5 min CTV			1 h CTV			Control
		CPM[a]	MDD[b]	CPM	MDD	RPS[c]	CPM	MDD	RPS	CPM
1	1.3	78	12	40	13	49	26	15	67	0
2	1.9	64	12	40	15	37	34	15	47	4
4	4.3	54	12	41	17	25	30	14	45	0
6	8.2	27	16	26	15	0	32	15	0	0

[a]CPM = cumulative percent mortality
[b]MDD = mean day to death
[c]RPS = relative percent survival when compared to mock-treated group

Table 3. *Oncorhynchus mykiss*. Neutralizing antibodies to IHNV detected in rainbow trout serum following exposures to CTV, IHNV or both viruses. Five pooled serum samples were obtained from fish in each group at 4 to 8 wk post challenge with IHNV and assayed for the PNT

Treatment	Weeks post CTV exposure			
	2	4	6	Control
CTV — 5 min	1280	640	320	<20
	1280	640	640	<20
	1280	2560	640	<20
	1280	640	320	<20
	1280	640	640	<20
CTV — 1 h	2560	2560	640	<20
	2560	1280	640	<20
	2560	2560	2560	<20
	2560	1280	1280	<20
	640	1280	1280	<20
Mock treated	640	2560	320	<20
	1280	640	640	<20
	2560	2560	160	<20
	2560	1280	320	<20
	2560	640	640	<20

the 2 treatment groups just 1 d prior to IHNV challenge indicated that CTV could be recovered (3/5 fish) from the CTV-exposed but not the mock-treated group (0/5 fish) or unexposed control group (0/5).

Trial 2

In the second trial, the duration of the protection afforded to IHNV challenge was examined as were the effects of 2 exposure times to CTV (5 min and 1 h). The protection provided was significantly greater ($p < 0.05$) in all groups exposed to CTV at Weeks 1, 2 and 4 when compared to mock-treated groups. Neither CTV group (5 min or 1 h) was significantly protected from IHNV challenge at 6 wk post CTV exposure (Table 2).

At 1 wk, the 1 h CTV exposure was significantly more protective than the 5 min exposure, but at 2 and 4 wk there were no significant differences between the protection afforded by CTV exposure for 5 min or 1 h. Based on the relative percent survival, the greatest protection (RPS = 70) was observed at 2 wk post CTV exposure in the 3 h CTV group in Trial 1 (Table 1). A similar response (RPS = 67) was observed among trout that had received prior exposures for 1 h when they were challenged with IHNV at 1 wk post CTV exposure in Trial 2 (Table 2). Significant protection ranging from 37 to 49 RPS was observed in the 5 min CTV-exposed groups challenged with IHNV at 1 and 2 wk and among their CTV 1 h counterparts at 1, 2 and 4 wk (Table 2). An examination of the neutralizing antibody response to IHNV among treatment groups at 4 to 8 wk post IHNV challenge showed that all treatment groups except the unchallenged controls were producing anti-IHNV antibody (Table 3) with titers ranging from 160 to 2560.

Trial 3

In the third trial, an RPS of 50 was observed for fish previously exposed to CTV and then challenged with IHNV (Table 4). Antibody titers observed in the serum of these fish at 5 wk post IHNV challenge were significantly greater ($p = 0.007$) than those from an equal

Table 4. *Oncorhynchus mykiss*. Susceptibility of rainbow trout to infectious hematopoietic necrosis virus (IHNV) following exposures to the cutthroat trout virus (CTV) and resulting serum neutralizing antibody titers prior to a second challenge with IHNV. Rainbow trout (2.6 g) were treated with either culture media from uninfected cells (mock) or cutthroat trout virus for 1 h. Fish were challenged with IHNV strain 220-90 at a dose of approximately 10^4 pfu ml^{-1} (primary challenge) or 10^5 pfu ml^{-1} (second challenge) for 1 h before resuming the flow of 15°C water into the aquaria. Mean serum antibody titers were determined for 22 two-fish pools from both IHNV exposed groups and 11 two-fish pools from each of the other control groups at 5 wk post primary challenge with IHNV. Titers expressed as the reciprocal of dilution with 50% or more reduction in plaques. The antibody titer of CTV/IHNV† was significantly higher ($p = 0.007$) than the CTV-mock group following IHNV exposure

	Treatment						
	CTV-mock/IHNV		CTV/IHNV		CTV		Control
	CPM[a]	RPS[b]	CPM	RPS	CPM	RPS	CPM
Primary challenge	32	–	16	50	–		–
(Fish wt. 2.6 g)							
Anti-IHNV antibody titer ml^{-1}	240		640†		<20		<20
Second challenge	3	93	0	100	28	28	40
(Fish wt)	(7.1 g)		(6.1 g)		(6.3 g)		(6.1 g)

[a]CPM = cumulative percent mortality
[b]RPS = relative percent survival when compared to mock-treated group

number of trout challenged only with IHNV (mock CTV). There was no evidence of antibodies in the serum of fish not previously exposed to IHNV.

Both treatment groups (CTV, mock CTV) receiving previous challenges with IHNV were protected against a second challenge of IHNV (Table 4). The mortality among the controls for those 2 groups (CTV, mock CTV) not initially exposed to IHNV was less than that obtained in most studies because these trout had increased in size and therefore resistance to IHNV (LaPatra et al. 1990). There was only 1 dead fish (1/25) among the CTV/IHNV control group (not rechallenged) and none in any other control group during the 3 wk period of the rechallenge experiment.

Virus recoveries from mortalities in all trials

As previously described, a portion of all dead fish were examined for the presence and concentration of IHNV in Trials 2 and 3 (LaPatra et al. 1993b). A recovery rate of 93% was achieved from all dead fish exposed to IHNV with the virus concentration averaging $10^{5.3}$ pfu g^{-1} of tissue. There was no IHNV detected among any fish from groups not exposed to IHNV.

Detection of antiviral activity

Cultures of AK leucocytes stimulated by CTV produced antiviral cytokine or interferon-like activity in inverse proportion to the dilution of virus used for induction. The levels of interferon-like activity induced by dilutions of CTV (MOI) at 1:32 (1.5), 1:64 (0.75) and 1:128 (0.37) were 509 ± 35.3, 312 ± 43.5 and 114 ± 37.1 $TCPD_{50}$ U ml^{-1}, respectively. The activity induced by CTV at a MOI of 1.5 was similar to the activity induced by IHNV at a MOI of 2.8 (476 ± 132 $TCPD_{50}$ U ml^{-1}). Comparisons of antiviral activities in supernatants of CTV-stimulated cultures after 24 h (1118 ± 222 $TCPD_{50}$ U ml^{-1}) and 72 h (1598 ± 211 $TCPD_{50}$ U ml^{-1}) indicated that much of the activity was produced in the first 24 h following CTV exposure.

DISCUSSION

Interference between heterologous viruses has been described *in vitro* and *in vivo* for numerous animal viruses (Holland 1985). With fish, viral interference has been demonstrated *in vitro* (Nicholson & Dunn 1974, Nicholson & Dexter 1975, Hedrick & Fryer 1982) and although double viral infections with IPNV and IHNV have been reported *in vivo* (Mulcahy & Fryer 1976, LaPatra et al. 1993a), only recently has the potential interference between these viruses in the host been described (de Kinkelin et al. 1992). Building on these initial observations, and using an avirulent picornavirus-like agent (CTV) as the stimulant, we have found that a strong protective response to the virulent rhabdovirus IHNV can be induced. Induction of the protective response is rapid but of limited duration suggesting that cytokines, as demonstrated *in vitro* with isolated anterior kidney cells, may be a major mechanism by which the antiviral state is achieved. Prior CTV exposure increased survival by as great as 70% and lengthened the mean day to death (MDD) as demonstrated by results from the first trial (Table 1). Significant protection was observed in Trials 2 and 3 with RPS values from 25 to 67.

The duration of the protection afforded by CTV appeared to be greatest during the first several weeks following exposure, as demonstrated in Trial 2 where protection was lost by 6 wk following exposure to CTV (Table 2). The protective state induced by CTV persists for at least 4 to 5 wk as shown in Trial 2 and again in Trial 3. The basis of this protective state is unknown but is presumably related to replication of CTV. Prior studies showed that following exposure of trout to the virus, replication occurred in several internal organs for periods up to 4 wk (Hedrick et al. 1991). In addition, we have shown that other sites of CTV replication include the skin and gills (S. Yun unpubl. data), and it may be at this site that some local protection is afforded to the fish upon IHNV challenge.

Several possibilities for stimulating nonspecific immune functions can be postulated. These include stimulation of macrophages or nonspecific cytotoxic cells and interferon induction. Cytokine activity is central to these responses but unfortunately, in salmonids, many of these factors, particularly as they relate to viral pathogenesis, are poorly understood.

Interferon of the α and/or β type (type I salmonid IFN) has been detected both directly in serum or tissue extracts of virus-infected rainbow trout (de Kinkelin & Dorson 1973, de Kinkelin & Le Berre 1974, Dorson et al. 1992). In all of these cases active replication of viral pathogens such as rhabdoviruses (IHNV or viral hemorrhagic septicemia virus, VHSV), or birnaviruses (IPNV) was required for IFN induction. Induction of IFN, however, is not completely dependent on virus replication, as recent studies by Rogel-Gaillard et al. (1993) have demonstrated that glutaraldehyde-fixed monolayers of EPC cells infected with VHSV can induce the production of interferon-like activity from trout leukocytes.

The kinetics for the production of IFN has been correlated to the longevity of viral replication (de Kinkelin et al. 1982, Renault et al. 1991). Recently, Eaton (1990) demonstrated that synthetic oligomers

(poly inosinic:cytidylic acids) can induce IFN in salmonids which in turn may protect them from viral infections. We might presume then that if CTV replicates in trout tissues, as previously demonstrated (Hedrick et al. 1991), IFN may be produced and therefore provide protection to IHNV infection. The studies reported here demonstrate that in isolated AK cells, CTV is a potent stimulator of IFN-like activity. Similar experiments will need to be conducted to determine if circulating IFN activity can be detected in the serum of CTV-infected trout.

De Kinkelin et al. (1992) have described a similar resistance to that induced in our studies among rainbow trout that had acquired natural infections with IPNV prior to challenge with VHSV. It is known that IPNV can initiate chronic or persistent infections, perhaps for the lifetime of trout infected as alevins (Billi & Wolf 1969, Wolf 1988). In addition, IFN induction by IPNV has been described in juvenile rainbow trout (Dorson et al. 1992). In our studies, individual IFN-like activities of up to 1640 U ml^{-1} were detected following CTV stimulation of AK cells. These levels compare favorably to those reported by Renault et al. (1991) in rainbow trout (body homogenates) 14 to 21 d post infection with IPNV. The activity detected in AK cells in our study was only partially reduced (30 to 40‰) by treatment at pH 2 for 24 h as shown for IFN from trout stimulated by IPNV and IHNV (de Kinkelin & Le Berre 1974, Renault et al. 1991). The pH labile and nonlabile components of the factors involved in providing protection in our IFN assays suggest that more than 1 cytokine may be involved. Further studies on the nature of these components and their origin, particularly from sites outside of the kidney, will be required to further define the observed resistance. Curiously, CTV, IPNV and IHNV can replicate and presumably initially invade, via the epidermis (Yamamoto & Ke 1991, S. Yun unpubl. data). This may be the site where the protection is provided by the first virus to the second challenge virus. This may result from a localized production and activity of nonspecific humoral and cellular factors.

Nonspecific cellular factors may include macrophage or nonspecific cytotoxic cell activity. The role of these cell populations in fish is poorly understood, although a potential role for nonspecific cytotoxic cells or natural killer-like cells has been demonstrated in both rainbow trout and catfish. Evans et al. (1984) and Moody et al. (1985) were able to demonstrate that rainbow trout and channel catfish *Ictalurus punctatus*, respectively, possess subpopulations of leukocytes, present in the spleen, anterior kidney and peripheral blood that showed nonspecific, non-MHC restricted, cell killing capabilities for either virus-infected or parasitic cells. Greenlee et al. (1991) further demonstrated in rainbow trout that these host cells possessed killing mechanisms that involved both necrosis and apoptosis. Unfortunately, there have been no studies in fish that have examined how adventitious agents, such as CTV, might stimulate this response to provide the protection observed to subsequent challenge with IHNV

Regardless of the mechanisms involved with the induction of the observed resistance, the study of how agents (such as CTV) can interfere with IHNV replication should provide a better understanding of what steps are critical to the natural resistance or susceptibility of salmonids to IHNV. Initial studies to induce resistance to VHSV infections with CTV have been successful (P. de Kinkelin unpubl. data). It is hoped that by studying the basis for the resistant state induced by avirulent CTV we may exploit similar mechanisms to induce transitory protection when immunization in very young fish is not possible or further stimulate the effectiveness of vaccines when administered to older fish.

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