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Anti-viral activity in four species of salmonids following exposure to poly inosinic:cytidylic acid

W. D. Eaton*

University of Alaska Fairbanks, Juneau Center For Fisheries and Ocean Sciences, 11120 Glacier Highway, Juneau, Alaska 99801, USA

Abstract: Pink, chum, and sockeye salmon and steelhead trout produced an anti-viral interferon-like response following intraperitoneal injections of the dsRNA synthetic polymer poly inosinic:cytidylic acid (Poly I:C). In all cases, maximum anti-viral titers were reached between 3 and 7 d post-injection with Poly I:C. Following injection with Poly I:C, chum and sockeye salmon were challenged with Infectious Hematopoietic Necrosis Virus (IHNV) and chum salmon with Erythrocytic Necrosis Virus (ENV). Fish pretreated with Poly I:C and then challenged with IHNV developed an anti-viral response which resulted in a decreased IHNV titer, lower cumulative mortalities, and a delay in virus replication when compared to untreated fish. The ENV could not be transmitted to chum salmon that had been pretreated with Poly I:C, but was successfully transmitted to untreated fish.

INTRODUCTION

Interferons are glycoproteins that induce cells to synthesize anti-viral proteins that non-specifically inhibit virus replication through several mechanisms (Pestka et al. 1987). The synthetic dsRNA polymer poly inosinic:cytidylic acid (Poly I:C) has been found to stimulate interferon production in primates and in cell culture (Giron et al. 1981, Lampson et al. 1981, Nakamura et al. 1982). Enhanced production of interferon has previously been achieved in the SSE-5, STE-137, KO6, and RTG-2 salmonid cell lines by the introduction of Poly I:C and has resulted in the protection of these cells against Infections Pancreatic or Hematopoietic Necrosis Virus (IPNV or IHNV) (MacDonald & Kennedy 1979, Tengelsen et al. 1989). de Kinkelin & Dorson (1973) and Dorson et al. (1975) showed that rainbow trout *Oncorhynchus mykiss* are capable of producing interferon in response to Viral Hemorrhagic Septicemia Virus (VHSV), and de Kinkelin et al. (1982) showed that exogenous interferon in trout has anti-viral activity.

The non-specific protective components of the fish immune system, such as interferon, can be effective against virus replication and should be considered as an essential component in the overall protective response of salmonids. Since interferon-induced inhibition of viral replication is not specific to any one virus, enhancement of this naturally occurring system could result in protection of fish against different viral pathogens. In addition, the interferon-induced anti-viral activity does not involve an antibody response and, presumably, fish need not be immunocompetent to acquire protection. Thus, the induction of the salmonid interferon system may provide a mechanism whereby fry can be protected against different viruses prior to the development of humoral protection.

A study was conducted to determine if steelhead trout (*Oncorhynchus mykiss*) sockeye (*O. nerka*), pink (*O. gorbuscha*), and chum (*O. keta*) salmon produce an anti-viral response following injection of Poly I:C; if sockeye and chum salmon are protected against IHNV; and if chum salmon are protected against erythrocytic necrosis virus (ENV) following injection of fish with Poly I:C.

MATERIALS AND METHODS

Fish. Steelhead trout (mean wt 15 g), sockeye (5 g), pink (1.3 g) and chum (1.5 g) salmon with no history of IHNV or ENV were received from the National Marine Fisheries Service fish hatcheries at Little Port Walter near Sitka, Alaska or Auke Creek in Juneau, Alaska.

* Present address: Biology Department, Malaspina College, 900 Fifth Street, Nanaimo, British Columbia, Canada V9R 5S5

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The fish were tested and found to be ENV and IHNV-free and were maintained in tanks containing 150 gal (570 l) of aerated, static, dechlorinated city water, chilled to 7 °C for all experiments. Ammonia levels were monitored and kept below 1 ppm total ammonia by water changes every 3 d.

Cell cultures and viruses. Stock cultures of RTG-2 cells were kindly provided by the Alaska Department of Fish and Game (ADF&G) Fish Pathology Laboratory. Cells were propagated at 15 °C using Eagles Minimal Essential Medium supplemented with 2.0 mM glutamine, 0.01 M HEPES buffer, and either 0 % (EMEM-0) or 7.5 % (EMEM-7.5) fetal bovine serum.

The IHNV strain used was originally isolated from sockeye salmon adults returning to Auke Lake, Alaska in 1978. The virus had been passed 6 times prior to this study and was frozen at −70 °C. The virus was also provided by the ADF&G Fish Pathology Laboratory. The ENV used came from liver, kidney, and spleen homogenates of Pacific herring *Clupea harengus pallasi* experiencing an epizootic due to ENV in Auke Bay, in June, 1989 (Meyers 1989). The liver, kidney, and spleen of 5 herring with cytoplasmic inclusions in >70 % of their erythrocytes were homogenized in STE buffer (10 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, pH 8.0) at a 1:10 dilution (w/v) and then frozen and thawed to lyse the cells. The cell debris was pelleted at 2000 × *g* for 10 min, and the supernatant collected and filtered through a 0.22 µm filter. The supernatant was used as inoculum for the ENV transmission study. A similar preparation was prepared from 5 uninfected herring and used as control supernatant.

Anti-viral activity in salmonids. Twenty sockeye, pink, and chum salmon and steelhead trout were injected by the intraperitoneal route with 50 µg g^{-1} Poly I:C (No. P-1530, Sigma Chemical Company) in STE buffer or with 0.2 ml of STE buffer. Five fish from each group were collected on 1, 3, 5, 7, 9, and 11 d post-injection. The liver, kidney, spleen, and heart were removed from each fish, pooled, and diluted 1:2 (w/v) in sterile STE. The tissues were then macerated and the cell debris pelleted at 2000 × *g* for 15 min. The supernatant was collected and diluted 1:5 (v/v) in an antibiotic mixture (0.5 mg ml^{-1} gentamicin, 1 mg ml^{-1} streptomycin, 50 IU ml^{-1} penicillin, 25 µg ml^{-1} fungizone) and incubated overnight at 10 °C. The samples were centrifuged again at 2000 × *g* for 15 min and the supernatant collected. In an attempt to destroy any anti-viral antibodies present in the samples, an aliquot of the supernatant was frozen at −70 °C for 48 h and thawed, heated to 56 °C for 30 min, treated with concentrated HCl to lower the pH to 2 for 12 h, treated with concentrated NaOH to raise the pH to 7.5, and then dialyzed against cold STE for 12 h. Another aliquot was similarly treated but was also treated with 20 µg ml^{-1} trypsin (Sigma Chemical Company) for 4 h at 37 °C, then dialyzed against cold STE for 12 h.

Serial 2-fold dilutions (v/v) of the treated samples were prepared (to 1:2560) in sterile STE and 1 ml of each dilution was added to replicate monolayers of RTG-2 cells propagated with EMEM-7.5 in 24-well plates (Falcon Laboratories). The cells were incubated for 16 h at 15 °C, at which time the samples were removed from the cells and the cells washed 3 × in sterile STE. The washed cells were then inoculated with approximately 500 $TCID_{50}$ of IHNV and incubated at 15 °C. Replicate wells, inoculated with sample and no virus, served as cytotoxicity controls. The cells were examined for signs of IHNV-induced CPE daily for 14 d, and the results were recorded. The anti-viral titer

Table 1. *Oncorhynchus gorbuscha, O. keta, O. nerka* and *O. mykiss*. Anti-IHNV (Infectious Hematopoietic Necrosis Virus) titer in pink, chum, and sockeye salmon and in steelhead trout tissues following intraperitoneal injections of fish with Poly I:C. The anti-viral titer is expressed as the reciprocal of the highest dilution of fish tissue homogenate which when added to RTG-2 cells completely inhibited IHNV-induced cytopathic effects

Inoculation groups	Anti-IHNV titer (d post-inoculation)						
	0	1	3	5	7	9	11
Pink salmon							
Prior to trypsin treatment	0	160	320	640	320	160	0
After trypsin treatment	0	0	0	0	0	0	0
Chum salmon							
Prior to trypsin treatment	0	0	320	160	160	0	0
After trypsin treatment	0	0	0	0	0	0	0
Sockeye salmon							
Prior to trypsin treatment	0	0	160	320	320	0	0
After trypsin treatment	0	0	0	0	0	0	0
Steelhead trout							
Prior to trypsin treatment	0	160	640	640	320	0	0
After trypsin treatment	0	0	0	0	0	0	0

was recorded as the reciprocal of the highest dilution of sample that resulted in complete inhibition of virus replication.

Virus challenges following exposure to Poly I:C. Sockeye and chum salmon were injected by the intraperitoneal (i.p.) route with 50 μg g^{-1} Poly I:C in STE buffer or with 0.2 ml STE buffer. Four days later, half of the fish in each group were injected i.p. with 4×10^3 $TCID_{50}$ of IHNV and the other half with 0.2 ml EMEM-0. Twenty fish from each of the variously treated groups of each species were held in separate tanks to determine the percent mortalities due to IHNV, and the IHNV titer in all mortalities was determined by $TCID_{50}$ assay as described by Eaton et al. (1989) in order to demonstrate that the cause of death was the virus. In addition, 3 live fish from each treatment group were sampled each week for 5 wk and the IHNV $TCID_{50}$ titer of each pooled 3-fish sample was determined.

Two groups of 30 chum salmon were injected with 50 μg g^{-1} Poly I:C in STE buffer or with 0.2 ml STE buffer. An additional 2 groups of 30 chum salmon were immersed for 3 h in water containing 400 μg ml^{-1} Poly I:C. At 4 d post-injection or post-immersion 3 of the variously treated groups of chum salmon were injected with 0.3 ml of the ENV and the other 3 groups were infected with control supernatant from Pacific herring, as described above. Five fish from each group were sampled weekly for 6 wk. An individual blood smear from each fish was made on a glass microscope slide and stained with Diff-Quik (Dade Diagnostics, Inc., Aguada, Puerto Rico 00602). Fifty microscopic fields on each slide were examined (40× objective lens) for the presence of intracytoplasmic erythrocytic inclusions characteristic of ENV. Liver and kidney samples were removed from fish with such inclusions and were processed for electron microscopic examination using standard methodologies.

RESULTS

All 4 species of fish demonstrated the ability to produce an anti-viral response following injections with Poly I:C, with the maximum anti-viral titer occurring 3 to 7 d post-injection (Table 1). The titrated anti-viral activity was stable at −70 °C, heat and acid-stable, non-dialyzable, and trypsin-labile. All cytotoxicity controls were negative and no anti-viral activity was evident in control fish injected with STE buffer only (data not shown).

Sockeye and chum salmon that were not treated with Poly I:C demonstrated the first measurable IHNV titers and IHNV-induced mortalities by the second week following IHNV injection (Table 2). The maximum IHNV titers in sockeye salmon occurred by 21 d post-injection, with maximum cumulative mortalities of 45 % occurring at 28 d. The maximum IHNV titers in chum salmon occurred at 21 d post-injection, with maximum cumulative mortalities of 40 % occurring at

Table 2. *Oncorhynchus nerka* and *O. keta*. Concentration of Infectious Hematopoietic Necrosis Virus (IHNV) in pooled liver, kidney, and spleen samples from live sockeye on chum salmon injected with Poly I:C or STE buffer then challenged after 4 d with IHNV (Auke Creek, Alaska isolate)

Salmon	Post-injection (d)	Treatment: IHNV injection[a] Titer[c]	IHNV injection[a] Mortalities[d]	Poly I:C + IHNV injection[b] Titer	Poly I:C + IHNV injection[b] Mortalities
Chum					
	7	0	0/20 (0 %)	0	0/20 (0 %)
	14	5×10^3	2/20 (10 %)	0	0/20 (0 %)
	21	3×10^5	4/20 (20 %)	6×10^3	1/20 (5 %)
	28	6×10^4	8/20 (40 %)	4×10^3	1/20 (5 %)
	35	1×10^3	8/20 (40 %)	2×10^2	2/20 (10 %)
Sockeye	7	0	0/20 (0 %)	0	0/20 (0 %)
	14	2×10^3	2/20 (10 %)	0	0/20 (0 %)
	21	3×10^5	4/20 (20 %)	0	0/20 (0 %)
	28	6×10^5	9/20 (45 %)	1×10^3	0/20 (0 %)
	35	5×10^4	9/20 (45 %)	3×10^2	0/20 (0 %)

[a] Fish were injected with 0.2 ml of STE buffer, then 4 d later injected with 4×10^3 $TCID_{50}$ IHNV
[b] Fish were injected with 50 μg g^{-1} of Poly I:C in STE buffer and 4 d later injected with 4×10^3 $TCID_{50}$ IHNV
[c] Concentration of IHNV is expressed as $TCID_{50}$ g^{-1} of tissue (kidney, liver, and spleen pooled from 3 live fish)
[d] Mortalities are expressed as cumulative mortalities over the course of the study. IHNV titers in individual mortalities ranged from 2×10^5 to 6×10^5 $TCID_{50}$ g^{-1} of pooled kidney, liver, and spleen

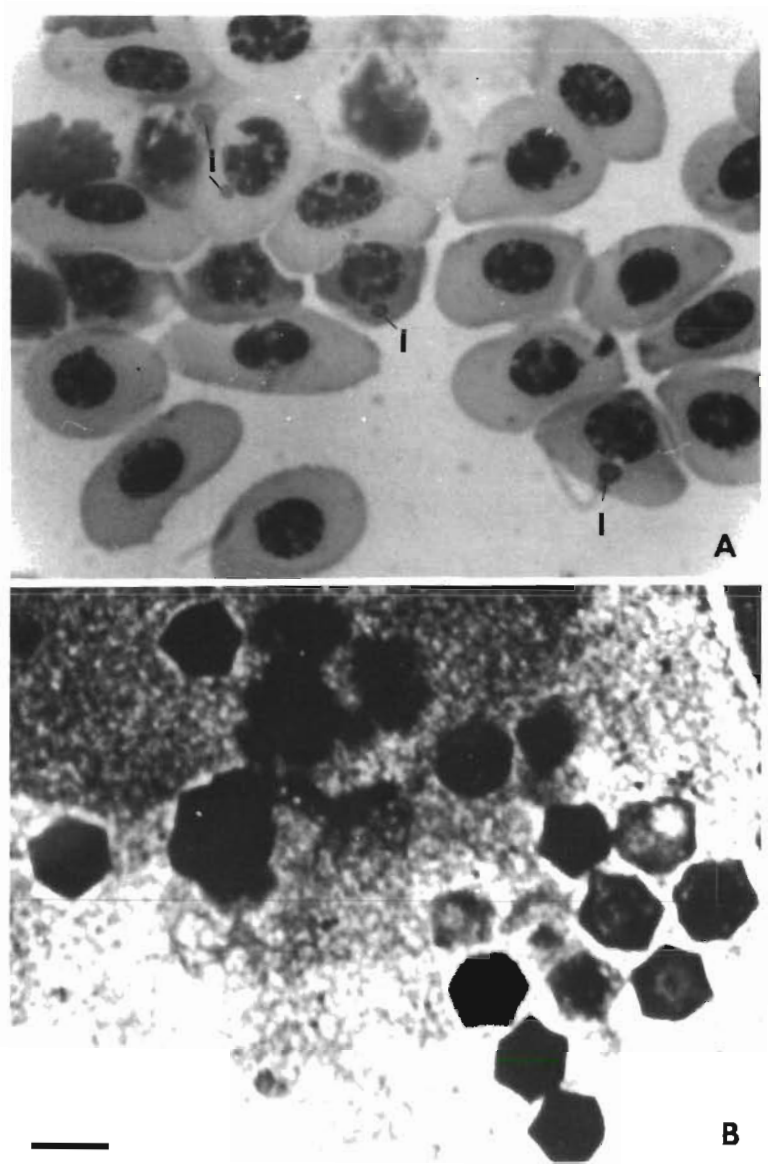

Fig. 1. *Oncorhynchus keta*. (A) Typical intracytoplasmic inclusion bodies found in the erythrocytes of chum salmon following injection with erythrocytic necrosis virus (ENV) from Pacific herring. I = inclusion bodies; × 1000. (B) Iridovirus-like particles, typical of ENV, found in the inclusion bodies of chum salmon following injection with ENV from Pacific herring. Scale bar = 200 nm

28 d post-injection. None of the control fish injected with Poly I:C and STE buffer or with STE buffer followed by injection of EMEM-0 developed IHNV infections or experienced any IHNV-related mortalities (data not shown).

Sockeye salmon injected with Poly I:C prior to exposure to IHNV demonstrated initial and maximum viral titers at 28 d post-injection with IHNV. No mortalities occurred in this group of fish. Chum salmon treated with Poly I:C prior to exposure to IHNV exhibited initial and maximum viral titers at 21 d post-exposure to IHNV and maximum cumulative IHNV-induced mortalities of 10 % by 35 d post-injection (Table 2).

Chum salmon controls injected with ENV after an initial treatment with STE buffer first exhibited cytoplasmic inclusions in the erythrocytes (Fig. 1) at 14 d post-exposure, the highest prevalence and intensity of infection occurring at 21 d post-injection (Table 3). At 21 and 28 d post-injection fish exhibiting such inclusions also had petechial hemorrhages at the base of the fins and at the vent. The erythrocytic inclusions were shown by electron microscopy to contain typical ENV-like Iridovirus particles that measured about 190 nm diameter (Fig. 1).

None of the chum salmon pre-treated either by injection or by waterborne exposure to Poly I:C prior to injection with ENV exhibited intracytoplasmic erythrocytic inclusions or any signs of morbidity (Table 3). None of the control fish injected with STE buffer or with Poly I:C followed by injections with the control supernatant developed intracytoplasmic erythrocytic inclusions (data not shown).

DISCUSSION

The results of this study show that an anti-viral response was developed in pink, sockeye, and chum salmon and in steelhead trout following injections with the potent interferon inducer Poly I:C. This anti-viral response provided protection against IHNV infection in sockeye and chum salmon and against ENV infection in chum salmon. In Poly I:C treated fish, the IHNV titer was about 10^2 $TCID_{50}$ lower at the peak of infection than in untreated fish. Similarly, in Poly I:C treated fish cumulative mortalities were significantly lower, and initiation of virus replication was markedly delayed, relative to that in untreated fish. The ENV also was not transmitted to fish pretreated either by injection or waterborne exposure to Poly I:C.

MacDonald & Kennedy (1979) and Tengelsen et al. (1989) demonstrated that cell lines derived from steelhead or rainbow trout and sockeye or kokanee salmon are capable of producing an interferon or an interferon-like response following exposure to Poly I:C. Dorson et al. (1975) and de Kinkelin & Dorson (1973) demonstrated the ability of rainbow trout to produce interferon following exposure to VHSV and de Kinkelin et al. (1982) showed that exogenous interferon in trout induced an anti-viral response. Although not all of the tests described by de Kinkelin & Dorson (1973) for characterizing interferons were conducted in the present study, the induced anti-viral activity exhibited by the 4 species of salmonids was clearly interferon-like and not due to anti-viral antibodies. It was stable at −70 °C, heat- and acid-stable, non-dialyzable, and trypsin-labile. Further, because salmonids are known to produce interferon in response to virus infections and because certain salmonid cell lines are known to produce interferon in response to Poly I:C, an interferon-type response would not have been unexpected.

Regardless of the nature of the response, it is clear that salmonid fry of 1.5 g or more are capable of developing an anti-viral response following injection with Poly I:C that is not antibody-mediated. Further, the response is protective against at least one RNA virus (rhabdovirus) and one DNA virus (iridovirus). It also appears that waterborne exposure of salmon to

Table 3. Erythrocytic Necrosis Virus (ENV) in *Oncorhynchus keta* (chum salmon) following injection of or waterbath exposure to Poly I:C or injection with STE buffer 4 d prior to injection with ENV supernatant derived from ENV-infected Pacific herring

	Days[a]	Inclusions[b]	% of erythrocytes with inclusions	Morbidity[c]
Chum salmon[d] (STE + ENV)	7	0/5	0 %	0/30
	14	1/5	< 1 %	0/25
	21	2/5	14.1 %	2/20
	28	2/5	8.6 %	2/15
	35	1/5	2.0 %	0/10
	42	0/5	0 %	0/5
Poly I:C + ENV[e] (injection)	7	0/5	0 %	0/30
	14	0/5	0 %	0/25
	21	0/5	0 %	0/20
	28	0/5	0 %	0/15
	35	0/5	0 %	0/10
	42	0/5	0 %	0/5
Poly I:C + ENV[f] (bath exposure)	7	0/5	0 %	0/30
	14	0/5	0 %	0/25
	21	0/5	0 %	0/20
	28	0/5	0 %	0/15
	35	0/5	0 %	0/10
	42	0/5	0 %	0/5

[a] Days post-injection with ENV or control supernatant
[b] Presence of intracytoplasmic erythrocytic inclusions
[c] Morbidity over course of study. Morbidity defined by the presence of petechiation at the base of fins and at the vent
[d] Chum salmon were injected with 0.2 ml STE buffer and 4 d later with ENV supernatant derived from ENV-infected Pacific herring
[e] Chum salmon were injected with 50 μg g^{-1} Poly I:C in STE buffer and 4 d later with ENV supernatant derived from ENV-infected Pacific herring
[f] Chum salmon were immersed for 3 h in water containing 400 μg ml^{-1} Poly I:C and 4 d later were injected with virus supernatant derived from ENV-infected Pacific herring

Poly I:C elicits the anti-viral response. Administration of Poly I:C by the water-bath route is a more logistically and economically feasible means of inducing an interferon response in fish on a large scale than the injection method. The Poly I:C waterborne exposure method of inducing an interferon-like response needs, however, to be evaluated for its ability to protect very young salmonids against viral pathogens such as IHNV or IPNV. This is important because many hatchery-reared salmonids are lost to viral infections at the stage before they have become immunocompetent.

Although the results of this study are encouraging, more work is needed to determine the efficacy of alternative inducers of the interferon-like response in fish. Poly I:C is expensive, and exposure of fry to Poly I:C on a hatchery-wide scale (even using a bath exposure) may be cost-prohibitive. In addition, Poly I:C has not been licensed for use as an anti-viral agent on food fish. Giron et al. (1981) used a combination of Amphotericin B, insulin and Poly I:C to induce an interferon response in mammalian tissue culture cells. This approach for interferon induction in fish warrants investigation. It is also necessary to determine if an anti-viral response can be induced in sac fry through bath exposures to an inducer. Finally, it would also be interesting to determine if injections of broodstock with inducer would confer any anti-viral protection to progeny fish.

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