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Involvement of rainbow trout leucocytes in the pathogenesis of infectious hematopoietic necrosis

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ABSTRACT: Rainbow trout *Oncorhynchus mykiss* leucocytes were tested for their ability to support replication of infectious hematopoietic necrosis virus (IHNV). Viral replication occurred *in vitro* using leucocytes cultured from peripheral blood, kidney, and thymus where viral titers peaked at 2 to 4 d post-inoculation. Leucocytes collected from trout following waterborne challenge with IHNV were co-cultured on EPC cell monolayers. These assays detected IHNV in leucocytes infected *in vivo* as early as 6 h post-exposure before the challenge virus had undergone replication. These data showed that leucocyte populations could serve as target cells in the initial phase of IHNV infection.

KEY WORDS: Leucocytes · Rainbow trout · IHNV

INTRODUCTION

Infectious hematopoietic necrosis (IHN) is an acute disease of salmonid fish caused by the rhabdovirus, infectious hematopoietic necrosis virus (IHNV). Viral replication occurs within a variety of fish tissues (Wolf 1988, Yamamoto et al. 1990) resulting in the presence of virus in body fluids (Mulcahy et al. 1982) and mucus (LaPatra et al. 1989c). Hematopoietic tissues of the kidney and spleen are particularly susceptible to fish rhabdoviruses such as spring viremia of carp virus (Ahne 1978), viral hemorrhagic septicemia virus (VHSV; de Kinkelin et al. 1979) and IHNV (Yasutake & Amend 1972) and these rhabdovirus infections often result in a leucopenia (Wolf 1988). The detection of IHNV is routinely accomplished by inoculating susceptible cell cultures with homogenates of hematopoietic tissues or with reproductive fluids (Amos 1985). The presence of IHNV antigens in cell cultures and in tissues infected with the virus has been demonstrat ed by immuno-fluorescence (Arnzen et al. 1991, LaPatra et al. 1989b) and by immunohistochemical staining (Yamamoto et al. 1990, 1992, Drolet et al. 1993). In addition to leucocytes, hematopoietic tissues are composed of several cell types which appear to be important sites of virus replication (Yasutake & Amend 1972, Wolf 1988, Yamamoto et al. 1990).

The evidence supporting a close relationship between rhabdovirus infections and fish leucocytes remains largely indirect; however, experimental depletion of leucocytes *in vivo* leads to a decrease in the susceptibility of rainbow trout *Oncorhynchus mykiss* to VHSV (Chilmonczyk & Oui 1988). Cells expressing VHSV antigens have been detected among blood leucocytes of trout surviving experimental VHSV infection (Enzmann 1981) and cytopathic effects were observed in mitogen-stimulated kidney leucocytes infected with VHSV *in vitro* (Estepa & Coll 1991). Cells, many of which displayed the morphology of leucocytes, have been collected from the ovarian fluid of several species of salmonid fish from regions where IHNV is enzootic (Mulcahy & Batts 1987, LaPatra et al. 1989a). When placed into culture, these cells were shown to produce IHNV.

Viruses have been found associated with lymphoid cells from human and animal species. Lymphocytes can either be restrictive for virus replication or can harbor virus in a latent phase (Wheelock & Toy 1973). Experimentally, lymphocytes have been persistently infected *in vitro* with vesicular stomatitis virus (Epstein et al. 1966) and cytomegalovirus (St. Jeor & Weisser 1977). The purpose of the present study was to determine the ability of IHNV to infect and replicate in purified rainbow trout (RBT) leucocytes *in vitro* and to determine if RBT leucocytes could serve as target cells for initial infection of IHNV *in vivo*.

MATERIALS AND METHODS

Cell lines and virus. *Epithelioma papulosum cyprini* (EPC) cells (Fijan et al. 1983) were grown at 15°C in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 12 mM Tris buffer. The Western Regional Aquaculture Center (WRAC) reference isolate of IHNV was used in experimental infections.

Fish. Experimental infections and leucocyte collections were performed using IHNV-free rainbow trout having a mean weight of 200 g for *in vivo* infection and 20 g for *in vitro* infections.

Leucocyte populations. Leucocytes, as judged by staining and morphologic criteria, were collected from blood, kidney, and thymus. Cells from anterior kidney and thymus were obtained by forcing the tissues through a stainless steel mesh (Sigma) in cold, serum-free MEM. Leucocytes were isolated and collected after centrifugation (1200 × *g*, 15 min) through a Ficoll hypaque cushion as previously described (Chilmonczyk 1978). Peripheral blood was obtained from the caudal vein and diluted 10-fold using serum-free MEM. Blood leucocytes were then recovered by the same procedure as described for kidney and thymus. Leucocytes were cultured in MEM supplemented with 10% FBS (MEM-10).

Experimental infections. *In vivo* infections were induced in rainbow trout by exposing the fish to 4×10^4 plaque-forming units (PFU) ml^{-1} of IHNV. Fish were kept for 1 h in aerated static water and then held in pathogen-free water until sampling. Fish were sacrificed at 6, 10, 12, and 18 h post-exposure and leucocytes collected from blood, kidney, and thymus.

In vitro infections were performed on leucocytes pretreated with polyethylene glycol (Batts & Winton 1989). Cells were inoculated at a multiplicity of infection (MOI) ranging from 1 to 5 and incubated for 1 h at 15°C. The cells were washed twice with MEM and resuspended with sufficient anti-IHNV rabbit polyclonal antiserum (PAb) or mouse monoclonal antibody (MAb) 5G3 to neutralize the unabsorbed virus. After 2 h at room temperature, the cells were washed twice and suspended in MEM-10 for virus production or co-cultivation assays.

Mitogenic stimulation. Two mitogens were used to stimulate the leucocytes. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Westphal method, Difco) and phytohemagglutinin (PHA-P, Sigma) were used at a final concentration of 100 µg ml^{-1} (LPS) and 50 µg ml^{-1} (PHA-P) of culture medium. The cells were incubated for 3 d in the presence of the mitogens to induce maximal DNA synthesis (Chilmonczyk 1978), washed, and *in vitro* infection performed as described above without anti-IHNV serum treatment.

Co-cultivation. Leucocytes collected from RBT following waterborne infection were plated onto fresh monolayers of EPC cells in 8-well multiplates and incubated at 15°C. Co-cultivation assays were performed according to a 2-step procedure. The first step consisted of removing the free leucocytes and culture fluid after co-cultivation for 7 d. The EPC cell monolayers were stained with a mixture of formalin and crystal violet to detect cytopathic effects and scored as positive or negative. These results were reported as the first co-culture. The leucocytes in the culture fluids were then pelleted by low-speed centrifugation (200 × *g*, 5 min) and the supernatant removed and frozen at −80°C until the virus titer was determined by plaque assay. These results were reported as the first plaque assay. The second step consisted of resuspending the cell pellet in MEM-10 and layering the leucocytes onto fresh EPC cell monolayers. After 7 d incubation, the EPC cell monolayers were stained as before and the results reported as the second co-culture. The culture medium and free leucocytes in the second culture were given 3 freeze-thaw cycles, centrifuged, and the virus titer in the supernatant determined by plaque assay. These results were reported as the second plaque assay.

Immunofluorescence. Indirect immunofluorescence was used to detect IHNV antigens on the surface of infected leucocytes. The leucocytes were washed twice in MEM, pelleted, and resuspended in 200 µl of 1% anti-IHNV PAb diluted in MEM. Following a 30 min incubation at 0°C, cells were washed twice in MEM, then incubated in 200 µl of 1% goat anti-rabbit IgG-FITC conjugate (Sigma) in MEM for 30 min at 0°C. After 2 washes, the pellet was resuspended in 1 drop of MEM and transferred to a microscope slide for fluorescent count. In each sample, an average of 20 microscope fields were observed by alternating observations using UV or bright light. Consequently, approximately 200 cells (thymus) or 400 cells (blood, kidney) were counted and the percentage of cells with membrane fluorescence was recorded.

Plaque assays. Quantification of IHNV was by plaque assay using EPC cells incubated at 15°C for 7 d as described by Batts & Winton (1989).

RESULTS

Replication of IHNV in trout leucocytes

In vitro infections

Leucocytes collected from blood, kidney, and thymus of RBT that were infected with IHNV for 1 h and treated with anti-IHNV sera (PAb or MAb) had considerably decreased the IHNV background (Day 0) titers. As shown

Table 1. Replication of infectious hematopoietic necrosis virus in rainbow trout leucocytes cultured *in vitro*. Cells collected from kidney (K), blood (B) or thymus (T) were infected with IHNV at multiplicities of infection (MOI) ranging from 1 to 5. After virus adsorption, cells were incubated with mouse monoclonal antiserum (MAb), rabbit polyclonal antiserum (PAb) or left untreated (U). Incubations were performed with or without (•) agitation. ND: not determined

Trout	MOI	Ab	Days post-infection					
			0	1	2	3	4	7
1 K	4	U	[a]3.8×10^{7}[a]	ND	1.2×10^{8}	1.8×10^{8}	ND	ND
1 B	2	U	7.3×10^{6}	ND	ND	5.8×10^{7}	ND	ND
2 K	1	U	4.3×10^{6}	ND	ND	3.4×10^{7}	ND	9.7×10^{6}
3 K	1	MAb•	1.1×10^{6}	ND	1.2×10^{7}	8.8×10^{7}	ND	ND
3 K	1	PAb•	0.8×10^{6}	ND	1.2×10^{7}	5.5×10^{7}	ND	ND
3 B	1	MAb•	0.8×10^{6}	ND	4.7×10^{6}	1.0×10^{9}	ND	ND
3 B	1	PAb•	0.5×10^{6}	ND	3.5×10^{6}	1.4×10^{7}	ND	ND
4 K	1	U	4.0×10^{7}	ND	ND	8.1×10^{7}	9.7×10^{8}	ND
5 K	1	PAb	1.0×10^{4}	ND	ND	1.6×10^{8}	4.7×10^{7}	6.7×10^{7}
6 K	5	MAb	7.0×10^{3}	ND	5.2×10^{5}	4.1×10^{5}	ND	3.6×10^{5}
6 B	5	MAb	1.0×10^{3}	ND	3.7×10^{5}	5.6×10^{5}	ND	3.5×10^{5}
7 K	5	MAb	5.5×10^{3}	4.7×10^{5}	9.1×10^{5}	5.6×10^{5}	4.8×10^{5}	2.8×10^{5}
7 B	5	MAb	7.0×10^{3}	6.7×10^{5}	1.5×10^{6}	1.1×10^{6}	9.0×10^{5}	5.8×10^{5}
8 T	5	MAb	2.0×10^{4}	ND	2.0×10^{5}	3.0×10^{5}	ND	6.5×10^{5}
9 K	5	MAb	2.0×10^{3}	6.0×10^{5}	1.0×10^{6}	4.4×10^{5}	1.0×10^{6}	7.0×10^{5}
9 B	5	MAb	9.5×10^{3}	1.0×10^{6}	4.1×10^{6}	5.3×10^{6}	1.5×10^{6}	9.6×10^{6}
10 T	5	MAb	2.0×10^{4}	2.0×10^{5}	5.6×10^{5}	1.7×10^{5}	3.2×10^{5}	3.0×10^{5}

[a]IHNV titers are expressed as PFU ml^{-1} determined at 0, 1, 2, 3, 4, and 7 d post-infection

in Table 1, IHNV titers increased in the leucocyte cultures indicating that viral replication occurred *in vitro*. Virus production peaked at 2 to 4 d post-inoculation and declined thereafter as dead cells (as judged by the trypan blue exclusion test) appeared in the culture. While the increase in IHNV titer was less than typically observed in monolayer cultures of established fish cell lines, the viral titers in the leucocyte cultures showed a consistent increase of 3- to 1000-fold above the initial (Day 0) titer.

Similar experiments were performed using leucocytes that were stimulated with either T-cell (PHA-P) or B-cell (LPS) mitogens. Cells were mitogen-treated either 1 to 3 d before or immediately following *in vitro* infection. In both experimental designs, no significant decrease or increase of IHNV titer was measured compared to the IHNV titers produced by the non-activated leucocytes (Table 2).

In vivo infections

We used an experimental waterborne challenge to determine if leucocytes could serve as target cells for initial infection of RBT with IHNV. Infection of the leucocytes with IHNV was demonstrated by co-cultivation on EPC monolayers. As shown in Table 3, the total number of samples from which IHNV could be recovered increased regularly with additional time post-infection. At 6 h post-infection, IHNV could only be detected in the blood and kidney leucocytes of 2 fish from a total of 22 that were sampled. However, at 18 h post-infection, 70% of the samples harbored IHNV.

In these experiments, when sampling occurred early after infection (6 h), the virus could only be detected

Table 2. Replication of infectious hematopoietic necrosis virus in mitogen-stimulated rainbow trout leucocytes cultured *in vitro*. Kidney, blood and thymus leucocytes were collected from a rainbow trout and cultured for 3 d in the presence or absence (unstimulated) of the mitogens phytohemagglutinin (PHA) or lipopolysaccharide (LPS) prior to infection with IHNV. IHNV titers were determined at 0, 1, 2, 3, and 4 d post-infection and are expressed as PFU ml^{-1}. Data are representative of 5 experiments (5 trout). ND: not determined

	Days post-infection				
	0	1	2	3	4
Kidney cells					
Unstimulated	3.5×10^{7}	3.6×10^{7}	6.0×10^{7}	1.4×10^{8}	1.1×10^{8}
PHA	3.4×10^{7}	4.0×10^{7}	5.8×10^{7}	1.6×10^{8}	9.1×10^{7}
LPS	3.8×10^{7}	4.1×10^{7}	6.2×10^{7}	1.2×10^{8}	9.4×10^{7}
Blood cells					
Unstimulated	4.0×10^{7}	6.2×10^{7}	8.3×10^{7}	2.1×10^{8}	1.2×10^{8}
PHA	3.8×10^{7}	5.4×10^{7}	9.0×10^{7}	1.8×10^{8}	1.4×10^{8}
LPS	3.8×10^{7}	5.1×10^{7}	1.1×10^{8}	1.9×10^{8}	ND
Thymus cells					
Unstimulated	3.8×10^{7}	ND	1.3×10^{8}	1.4×10^{8}	7.6×10^{7}
PHA	4.0×10^{7}	ND	7.7×10^{7}	1.5×10^{8}	8.4×10^{7}
LPS	3.8×10^{7}	ND	8.9×10^{7}	1.3×10^{8}	7.2×10^{7}

Table 3. Infection of leucocytes following waterborne challenge of rainbow trout with IHNV. Leucocytes were obtained from blood or kidney of rainbow trout at 6, 10, 12, and 18 h following waterborne challenge of the fish with IHNV Co-cultivation assays were used to detect infected leucocytes. Values represent the no. of fish positive/no. of fish examined at each sampling time

	Sampling time post-infection			
	6 h	10 h	12 h	18 h
First co-culture	0/22	0/14	3/30	9/20
First plaque assay	0/22	3/14	6/30	9/20
Second co-culture	1/22	3/14	6/30	14/20
Second plaque assay	2/22	3/14	9/30	14/20
Total positive cultures	2/22	3/14	9/30	14/20
Percent positive cultures	9	21	30	70

after the second co-cultivation on EPC cells. At the other sample periods, the second co-cultivation step greatly improved the detection of infected leucocytes. This was probably due to the very low level of infectious virus present in the leucocytes at the time of the early sampling. In leucocytes collected at 6 and 10 h post-infection, the whole volume (2 ml) of each co-cultivation supernatant was titered by plaque assay and only 3 to 70 PFU were measured in each of the positive samples. For cells collected at 12 and 18 h post-infection, the number of IHNV particles had increased to 4×10^2 to 5×10^3 PFU per sample.

Quantification of IHNV produced by leucocytes

To assess the number of PFU produced by IHNV-infected leucocytes, a comparison was made between the number of cells expressing IHNV antigen (determined by immunofluorescence) and the virus titer of the same culture. At daily intervals following *in vitro* infection, leucocyte viability, IHNV titers of the cultures, and percentage of leucocytes expressing IHNV antigen were determined (Table 4). From these data, it was possible to estimate the number of PFU produced by each infected cell. Virus production increased in the culture and each infected leucocyte could produce from 1 to 145 PFU. As expected, cell viability regularly decreased during the course of *in vitro* infection.

The percentage of cells expressing IHNV-specific fluorescence was noticeably higher in the leucocyte cultures derived from thymus than from blood or kidney; however, no direct relationship could be established between the percentage of specific fluorescent cells and the IHNV titers. The yield of infective particles produced by the thymic leucocytes was inversely related to the number of cells expressing IHNV antigen. If each of the latter cells was assumed to be synthesizing infectious virus, the maximum number of infectious particles produced by each thymic leucocyte was very low (2 to 3 PFU $cell^{-1}$). Blood and kidney cells appeared to be more efficient at replicating IHNV, producing 145 and 12 PFU $cell^{-1}$, respectively.

Characterization of leucocytes supporting IHNV replication

Several cell types were detected expressing IHNV antigen. These cells exhibited the typical morphology of small lymphocytes, monocytes, and polymorphonuclear cells.

DISCUSSION

Our results showed that IHNV replicated in leucocyte populations derived from blood, kidney, and thymus of rainbow trout and that diverse types of leucocytes were involved. Some of the cells replicating IHNV exhibited the morphology of small lymphocytes; nevertheless, addition of mitogen to the cultures did not affect the virus titer. Cytopathic effects were observed by Estepa & Coll (1991) following VHSV infection of *in vitro* stimulated RBT lymphocytes. In mammals, virus replication in lymphocytes has largely been reported in activated cells. Cells undergoing blastogenesis under either specific or non-specific (mitogenic) stimulation have been shown to replicate herpes virus (Kleinmann et al. 1972), measles virus (Joseph et al. 1975), and vesicular stomatitis virus (Edelman & Wheelock 1966, Schmidt & Woodland 1990).

The *in vitro* exposure of RBT leucocytes to IHNV resulted in establishment of infected cultures characterized by great differences in virus production. Routinely, we consider that 1 EPC cell can produce at least 400 PFU. Our data showed that leucocytes produced fewer virions (145 or fewer PFU) per cell; however, this type of experiment is often hampered by a variety of factors. The collection methods allow only a portion of the peripheral blood or tissues to be sampled and the purification techniques lead to the loss or damage of a portion of the leucocyte population. Additionally, *in vitro* culture conditions are far from ideal and may induce alterations in cell structure and function that can markedly affect experiments on virus-leucocyte interactions. Nevertheless, the significant increase in IHNV titers in cultured leucocytes and the demonstration of IHNV antigens on the surface of infected cells establishes that these cells are susceptible to infection by IHNV and capable of supporting the replication of the virus *in vitro*.

Table 4. Production of IHNV by rainbow trout leucocytes cultured *in vitro*. Cells were harvested from blood, kidney, and thymus and the number of infectious plaque-forming units (PFU) produced by each infected cell was estimated by comparing the virus titer of the cultures with the number of cells showing IHNV antigen by immunofluorescence (FITC)

	Days post-infection						
	0	1	2	3	4	6	8
Blood							
Viable cells ($\times 10^6$)	4.75	3.75	3.75	3.25	2.75	2.0	2.25
Percent positive by FITC	0	1.6	4.2	3.5	3.6	3.3	2.3
IHNV titer	9.5×10^3	1.0×10^6	4.1×10^6	5.3×10^6	1.5×10^6	9.6×10^6	1.4×10^5
Number PFU/positive cell	0	16.6	26.1	46.9	15.1	145	2.7
Kidney							
Viable cells ($\times 10^6$)	8	7.75	5.0	3.75	3.75	3.75	3.5
Percent positive by FITC	0	1.4	2.0	1.9	2.8	1.5	1.8
IHNV titer	2.0×10^3	6.1×10^5	1.0×10^6	4.4×10^5	1.0×10^6	7.0×10^5	3.7×10^5
Number PFU/positive cell	0	5.6	10	6.2	9.5	12.5	5.8
Thymus							
Viable cells ($\times 10^6$)	3.25	2.75	2.25	2.5	2.25	2.25	1.75
Percent positive by FITC	0	6.0	10.7	14.0	15.4	27.7	24.6
IHNV titer	2.0×10^4	2.0×10^5	5.6×10^5	1.7×10^5	3.2×10^5	3.0×10^5	2.7×10^5
Number PFU/positive cell	0	1.2	2.3	0.5	0.9	0.5	0.6

Our *in vitro* data were confirmed by results obtained from *in vivo* infections. The co-cultivation assays used for detection of IHNV-infected leucocytes appeared to be capable of detecting even the low rate of infectivity as occurred here with leucocytes collected from fish sampled early after waterborne infection. Although it did not allow an accurate estimation of the percentage of infected cells, the assay detected infective IHNV particles associated with leucocytes as soon as 6 h post-infection, illustrating that leucocytes play a role in the pathogenesis of IHN. At 6 h post-infection, the initial virus replication cycle could not have been completed and the IHNV in the leucocytes must have had its origin in the virus added to the water during the experimental waterborne infection. These data indicate that leucocytes are among the initial target cells for IHNV.

The mechanisms involved in the establishment and maintenance of IHNV infection in fish are not understood. While our data showed that limited replication of IHNV occurred among populations of RBT leucocytes, the titers were not sufficient to suggest that this represented an important source of total viral production in natural infections. Although our co-cultivation experiments showed that leucocytes may represent one of the cell types involved in primary infection with IHNV, it does not seem likely that the primary target cells for fish rhabdoviruses are restricted to leucocytes. In addition to mitogen-stimulated trout leucocytes, Estepa et al. (1992) reported that RBT macrophages supported replication of VHSV and Yamamoto et al. (1992) showed that epithelial cells in salmonid skin were capable of supporting replication of both IHNV and VHSV. Others cells from fish surface tissues are putative target cells for virus penetration and replication before spreading throughout the fish to susceptible tissues and organs and LaPatra et al. (1989c) detected IHNV in mucus from the external surface of salmonids. The external location and structure makes gills (Chilmonczyk & Monge 1980) and thymus (Chilmonczyk 1983) attractive sites for virus entrance into the fish. Both organs contain rich populations of leucocytes which may represent an easy method for disseminating the virus.

Our results showed that the percentage of cells expressing IHNV antigens was higher in thymus than in blood or kidney, but virus production appeared to be less efficient in the thymus than in the other organs. The cellular events responsible for the susceptibility of fish cells to become infected and to replicate IHNV are not yet elucidated. To improve our knowledge of the pathogenesis of fish rhabdoviruses, further experiments will be needed to identify more precisely the populations of leucocytes that first replicate the virus and to determine the mechanism(s) and exact site(s) for the entrance of IHNV into fish cells.

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