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In vitro INFECTION OF TROUT KIDNEY CELLS WITH INFECTIOUS PANCREATIC NECROSIS AND VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUSES

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Introduction

In rainbow trout, *Oncorhynchus mykiss*, both isolation (Swanson and Guillespie, 1982) and replication (Yu *et al.*, 1982) of infectious pancreatic necrosis virus (IPNV) and viral haemorrhagic septicaemia virus (VHSV)(Chilmonczyk and Oui, 1988), have been related to their respective virus presence in trout lymphoid organs *in vivo*.

In the present work, we have studied the *in vitro* infection of isolated trout leucocytes with IPN and VHS viruses. To maintain the trout leucocytes we employed the fibrin-clot technique as described before for mouse myelomas (Rueda and Coll, 1988) and adapted for trout leucocytes (Coll, 1990).

Materials and methods

Kidney cells

Trout were purchased from commercial farms (5-20 g body weight) after several annual tests indicated they were free of infectious pancreatic necrosis. The trout were cooled to 4°C and bled from the tail vein to reduce the blood content of the kidney. The anterior kidney (pronephros) was removed and cut into pieces in a Petri-dish. Clumps of cells were dissociated by passing the suspension through a 20 gauge needle. The cell suspension was decanted, centrifuged at 1000 g for 10 min and washed in cold cell culture medium. The cell concentration was determined with a haemocytometre and adjusted to about 2 x 10 5 round cells/ml. Mature erythrocytes, distinguished by their oval shape, were not included in the counts.

Cell culture in fibrin-clots

The cell culture medium (Flow Lab, Ayrshire, Scotland) was RPMI-1640 (Dutch modification) with 2 mM L-glutamine, 1 mM sodium pyruvate, 1.2 n, the fibrin clots were removed from the wells onto a 75 x 25 mm frosted-end glass slide with a spatula and stained with 0.025% toluidine blue in 0.01 M sodium borate, pH 8 for 10 min and washed in running tap water for 10 min (Rueda and Coll, 1988).

Cells were studied at 400 x. Four cultures were made for each experiment, one field was counted for each culture (about 30 cells per field) and averages were calculated.

A complete description of the technique is published elsewhere (Coll, 1990).

Viruses

The strains of viruses used were spanish isolates of IPN-140 (Jimenez *et al.*, 1988) or VHS-144 (Basurco and Coll, 1989), from rainbow trout. The viruses were incubated in either chinook salmon embryo or epithelioma papillosum cyprini cells, and added to the kidney cell cultures as supernatant from infected cell monolayers after complete cytopathic effect. Purified VHSV was obtained as described (Basurco and Coll, 1989). Purified viral samples (purified IPNV was a gift from Dr. R. Hedrick) were heated to 100°C for 10 min to inactivate the virus.

Titration of virus in EPC monolayers

To recover the virus after infection, several clots were pooled, broken with Pasteur pipettes, centrifuged and the supernatant titrated in EPC cell monolayers for the presence of virus by the

TCID 50 method (Basurco and Coll, 1989). Parallel cultures to which the viruses were added immediately before processing were used as controls.

Immunofluorescence

Immunofluorescence of IPNV-infected cells was carried out after fixing the clots with ethanol, using international polyvalent reference antisera (rabbit anti-IPNV, was gifted by Dr. de Kinkelin). The method was used as described previously (Jimenez *et al.*, 1988).

ELISA

Supernatant from virus infected leucocyte cultures was examined for the presence of IPNV or VHSV by the 2 monoclonal antibody-based ELISA sandwich as previously described (Dominguez *et al.*, 1990). Results of ELISA were expressed by the reciprocal of the dilution needed to give an absorbance of 0.25 (A492-620 nm). The supernatants of the infected cultures were titrated by 2-fold dilutions in duplicate.

Results

The types of trout kidney cells present in control noninfected cultures are shown in Figure 1. IPNV-infected cultures showed a small increase in the total number of trout kidney cells relative to the controls. In culture from one individual (of 5) fish there were abundant but small colonies (4-6 cells per colony) of the large-nucleated cells (Figure 1, IPNV). Parallel cultures to which heat-inactivated purified IPNV was added, did not show any differences with control non-infected cultures. IPNV could be recovered in EPC from the supernatant of the virus-infected cultures but the titre had decreased about 4-fold (Table 1). By ELISA, IPNV recovered from the infected cultures was about 100%. In immunofluorescence, the leucocytes from infected cultures showed no significant difference when compared with non-infected cultures.

In cells from 8 different trout, the kidney cells from the VHSV-infected cultures were either dead or had low survival rates. Most of the cells in these cultures were completely lysed (Fig. 1, VHSV). The extent of this lysis was dependent on the experimental conditions of the infection. For instance, by using both a lower dose of virus and a lower pH (gassing with CO2 during infection) some surviving cells remained. Parallel cultures to which heat-inactivated purified VHSV was added, did not show any

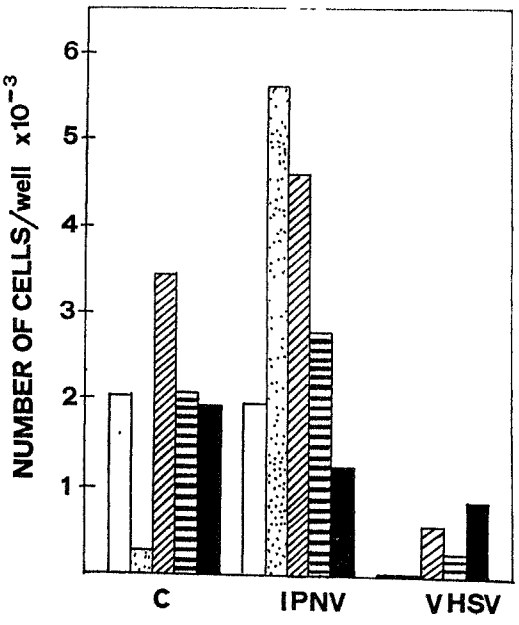
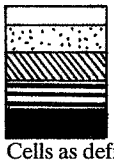


Figure 1. Cell types from rainbow trout kidney surviving infection with IPNV and VHSV *in vitro*.

Kidney cells were obtained from a 20 cm trout and plated at 2.4 x 104 cells/well. Cells were cultured at 20°C for 1 week, infected with 104 TCID50 of IPNV or VHSV per ml and cultured at 14°C for another week.



Lymphocytes
large-nucleated cells
multinucleated cells
eccentric-nuclei cells
adherent cells

Cells as defined previously (Coll, 1990).

Table 1. Replication of IPNV and VHSV in trout kidney cells.

Method	Time of virus addition	IPNV	VHSV
EPC monolayers	After incubation	1.6	1.6
	Before incubation	≦ 0.4	6.4
ELISA	After incubation	30	20
	Before incubation	30	45

Cell cultures were incubated for 1 week at 14°C. Viruses were added before the incubation or after the incubation. All the cultures were then processed simultaneously. Results are expressed as TCID50/ml x 103 after titration on EPC monolayers or as the dilution needed to give an absorbance of 0.25 after ELISA.

effects. Live VHSV could be recovered in EPC from the medium of the infected cultures with about a 4-fold increase in titre. By ELISA, the VHSV recovered from the infected cultures had increased about 2- fold in titre.

Discussion

The present experiments were undertaken to examine the *in vitro* infection of trout kidney leucocytes with salmonid viruses and to describe the morphology of the cell types affected.

VHSV lysed all the cell types described before in the fibrin-clot culture technique (Coll, 1990). No specific cell type could be found resistant to the infection in the cultures.

In sharp contrast, IPNV-infected cultures contained all the cell types morphologically intact. Since an association of the IPNV with leucocytes of trout has previously been demonstrated (Swanson and Gillespie, 1982; Yu *et al.*, 1982) it is possible that infection of trout leucocytes with IPNV *in vitro* results in the establishment of carrier-cells as demonstrated in lymphocytes from *in vivo* carriers (Knott and Munro, 1986). The leucocyte count tended to increase in the IPNV-infected cultures as compared with either non-infected controls or inactivated IPNV-infected cultures. Since we were unable to demonstrate highly fluorescent leucocytes or any virus titre increase after culture by TCID50 or ELISA, it is suggested that a specific stimulation of leucocytes could be taking place. This could be due to previous exposure to IPNV (positive evidence was obtained in at least 1 case) in the farm supplying the trout, in a similar way as has been shown for VHSV (Chilmonczyk, 1977). More research must be done with completely identified trout to solve this aspect. The failure of inactivated virus to induce specific stimulation could be due to the destruction of the required antigens by heating. The results obtained suggested that the trout leucocytes are not a lytic target for IPNV. The possible interferon production by the kidney cells to neutralize IPNV, is not very likely because the same cell cultures (from the same fish) were simultaneously infected with the IPNV or the VHSV. On the other hand, IPNV could be more susceptible to interferon than VHSV.

Summary

The *in vitro* effect of infected cultured rainbow trout kidney cells with viral haemorrhagic septicaemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV) was studied. VHSV destroyed all the trout kidney cells by replicating in them but IPNV did not replicate and caused no cell death, but instead tended to increase the number of cells. Specific stimulations of leucocytes from trout surviving natural IPNV infection could explain these results.

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