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# Infection of mitogen-stimulated trout leucocytes with salmonid viruses

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**Abstract.** The effects of infection with salmonid viruses on mitogen-stimulated cells and colonies from rainbow trout, *Oncorhynchus mykiss* (Richardson), kidney cell cultures have been studied using culture in clots of fibrin. Phytohaemaglutinin, concanavalin A and lypopolysaccharides from *Escherichia coli* have been used as mitogens. The viral haemorrhagic septicaemia (VHS) virus destroyed any mitogen-induced trout kidney colonies or cells, but the infectious pancreatic necrosis (IPN) virus caused no cell or colony death, and even increased their count in the cultures. The results suggested that the target for IPN virus replication is not any trout leucocyte but that among the possible targets of VHS virus are the different types of leucocytes found in the trout kidney and in their *in vitro* mitogen-stimulated colonies.

## Introduction

One of the greatest drawbacks in the development of fish viral vaccines is the incomplete state of knowledge of the immunology of the fish (Austin 1984). Despite the absence of any therapy for controlling viral diseases, investigations about cellular targets and/or immunity to the virus infections of fish have received very little attention. However, viral diseases, such as infectious pancreatic necrosis (IPN) or viral haemorrhagic septicaemia (VHS), do exist and jeopardize fish farms. On the other hand, fish are the most primitive animals to possess an adaptative immune system characterized by the existence of cells and IgM-like immunoglobulin (Sanchez & Coll 1989). Therefore, it is of interest to study how their immune cells react against viral infections.

In the rainbow trout, *Onchorynchus mykiss* (Walbaum), both isolation (Swanson & Gillespie 1982) and replication (Yu, Macdonald & Moore 1982) of infectious IPN virus or susceptibility to VHS virus (Chilmonczyk & Oui 1988), have been indirectly related to their respective virus presence in trout lymphoid organs. However, it is not clear, at present, whether or not any of the lymphoid cells are the targets of replication of these viruses.

The present authors report here the results of the *in vitro* infection with the IPN and the VHS viruses of kidney trout leucocytes after culturing them with mitogens. To maintain the trout leucocytes, the fibrin clot technique was used as described previously (Rueda & Coll 1988; Coll 1990).

## Materials and methods

### *Chemicals and reagents*

Medium RPMI 1640 (Dutch modification) and foetal calf serum were obtained from Flow Lab (Ayrshire, Scotland). Lyophilized human fibrinogen was obtained from A. B. Kabi (Stockholm, Sweden), reconstituted with distilled water, dialysed extensively against distilled water, ly-

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ophylized and kept at −20 °C in aliquots. Gentamicin was from Schering Corp. (Kenilworth, NJ, USA), amphotericin was from Flow Lab, thrombin was from Miles Lab (Elkhart, Indiana, USA), phytohemaglutinin (PHA) and concanavalin A (Con A) were from Flow Lab (Ayrshire, Scotland), *E. coli* lipopolysaccharide (LPS) was from Difco Lab (Detroit, MI, USA).

*Cells from trout kidney*

Trout were purchased from commercial farms (5–20 *g* body weight) after several tests indicated they were free of infectious pancreatic necrosis. The trout were held in 30-l aquaria with dechlorinated free-flowing water at 12–18 °C until used; they were cooled down to 4 °C and bled by the tail vein to reduce the blood content of the kidney. The anterior kidney (pronephros) was removed under sterile conditions and cut into pieces with scissors in a Petri-dish. Clumps of cells were dissociated by passing the suspension through a 20-gauge needle. The cell suspension was decanted briefly from the undissociated tissue, centrifuged at 1000 *g* for 10 min and washed in cold cell culture medium twice. The cell concentration was determined with a haemocytometer and adjusted to 200 000 round cells $ml^{-1}$. Mature erythrocytes, distinguished by their oval shape, were not included in the counts. The contamination with mature erythrocytes was lower than 10%. Cell viability, as determined by trypan blue exclusion, was higher than 80%.

*Cell culture in fibrin-clots*

The cell culture media was RPMI-1640 (Dutch modification) with 2 mM L-glutamine, 1 mM sodium pyruvate, 1·2 $\mu g\,ml^{-1}$ amphotericin, 50 $\mu g\,ml^{-1}$ gentamicin, 20 mM Hepes, 50 $\mu$M mercaptoethanol, 10% pretested foetal calf serum and 0·5% pooled rainbow trout serum. Fibrinogen was added to the medium just before use to a final concentration of 0·2 $mg\,ml^{-1}$. Thrombin was added to the wells to a final concentration of 2–4 NIH U $ml^{-1}$.

Cultures of leucocytes were always from individual fish to avoid any possible mixed leucocyte reactions.

The cell suspension was prepared, thrombin was added at 0·2–0·4 NIH U $well^{-1}$ in 2 $\mu$l volume, and 100 $\mu$l of the cell suspension was pipetted into each well of a 96-well plate (Costar, The Netherlands). After clotting occured (in about 30 s), the mitogens dissolved in sterile water were pipetted into each well in a maximum volume of 10 $\mu$l on the top of the clot. The plates were then sealed in a 20 × 12 cm plastic bag (Vaessen, Schoemaker Indtal S.A., Sant Boi de Llobregat, Barcelona, Spain), gassed with 5% $CO_2$ in air, sealed and incubated at 20 °C for one week, infected with viruses and incubated at 14 °C for one more week.

After incubation, the fibrin clots were removed from the wells onto a 75 × 25 mm frosted-end glass slide with a spatula. The clots were partially dehydrated by placing a rectangular piece of Whatman No. 1 filter paper on their surfaces. Then five drops of 1·7% glutaraldehyde in 0·01 M sodium phosphate, 0·15 M sodium chloride, pH 7·4 were added and allowed to stand for 10 min. The excess of glutaraldehyde was absorbed with filter paper. The papers were removed with gentle pressure and the fixed clots were washed in water and air dried. After drying, they were stained for 10 min in 0·025% toluidine blue followed by 10 min in running tap water. After air drying, the preparations were permanently mounted with Permount (Fisher Sci. Co., NJ, USA).

Colonies were scored with the aid of an ocular eye piece at a magnification of 100×. Cell and colony types were studied at 400×.

*Viruses*

The strains of viruses used, IPN-Sp (Jimenez, Marcotegui, San Juan & Basurco 1988) or VHS-144 (Basurco & Coll 1989), were both isolated from rainbow trout in Spain. The viruses were cultured in either rainbow trout gonad (RTG-2) or epithelioma papillosum cyprini (EPC) cells, respectively, and added to the cultures as supernatant from infected-cell monolayers after the cytopathic effect was complete (Jimenez *et al.* 1988). Purified VHS virus was obtained as described (Basurco & Coll 1989). Purified viral samples (purified IPN virus was a gift from Dr Ronald Hedrick) were heated to 100 °C for 10 min to inactivate the virus. Viruses were titrated by the $TCID_{50}$ method, as described by Basurco & Coll (1989).

*Infection of the fibrin-clot cultures*

Trout kidney cells were cultured with the mitogens for one week at 20 °C, then infected with $10^4$ $TCID_{50}$ in 10 $\mu$l per well (about 0·5 m.o.i.), sealed and gassed with 5% $CO_2$ in air, incubated for one more week at 14 °C, and finally fixed and processed as described above.

To recover the virus after infection, several clots were pooled, broken with Pasteur pipettes, centrifuged and supernatant and pellet titrated in cell monolayers for the presence of virus by the $TCID_{50}$ method (Basurco & Coll 1989).

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

## Results

The composition of trout kidney cells varied from trout to trout as studied at time 0 in the fixed and stained clots. The rounded cells varied between 70 and 90% of the total cells, the rest being the oval-shaped erythrocytes. The abundance of erythrocytes also depended on the completeness of the bleeding of the trout prior to the kidney extraction. About 10–20% of the rounded cells were of the erythrocytic lineage, as determined by their cytoplasm staining and/or the patched nuclei. The non-erythrocytic rounded (leucocytes) cells could be classified as lymphocytes, large nucleated cells, multinucleated cells, eccentric nuclei cells and adherent cells. Their abundance and characteristics are shown in Table 1.

**Table 1.** Definition of cell types and their composition in the kidney from rainbow trout*

| | Size ($\mu$m) | | Percentages ($n = 6$) | | |
|---|---|---|---|---|---|
| Cells | Cell | Nuclei | Averages | (S.D.) | Ranges |
| Lymphocytes | 6 | 5 | 35·1 | (17·4) | 11–57 |
| Large nucleated | 11 | 9 | 14·1 | (3·4) | 10–19 |
| Multinucleated | 11 | 6 | 31·8 | (15·0) | 11–55 |
| Eccentric-nuclei | 11 | 6 | 14·1 | (5·3) | 7–20 |
| Adherent | 30 | 10 | 5·0 | (3·3) | 2–11 |

* The trout were obtained in August, November, December, January, June and September.

The presence of mammaliam mitogens (PHA, ConA or LPS) in the cultures, stimulated both cells and colonies. Figure 1 shows the morphology of the colonies at low magnification both *in vivo* by phase contrast and after fixing and staining. The average size of the colonies was $44 \pm 12$ ($n = 30$). After 2 weeks in culture, the cells in the colonies were mostly of the eccentric-nuclei type.

Both eccentric-nuclei and multinucleated cells were the most abundant cells in the cultures with any of the mitogens employed (Fig. 2). Their count increased when the concentration of mitogens increased, either with an optimum of 2 $\mu$g of PHA ml$^{-1}$ for eccentric-nuclei cells and 10 $\mu$g of PHA ml$^{-1}$ for multinucleated cells, or with a continous increase from 0 to 100 $\mu$g of ConA ml$^{-1}$ and from 0 to 200 $\mu$g of LPS ml$^{-1}$. The strongest stimulator was PHA, followed by ConA and LPS; for example, to obtain an stimulation of 4000 eccentric-cells per well (four-fold with respect to controls without mitogens), about 0·2, 20 or 200 $\mu$g ml$^{-1}$ of PHA, ConA or LPS, respectively, were needed in the culture (Fig. 2). The count of colonies was dependent on mitogen concentration, showing an optimum at 2 $\mu$g of PHA ml$^{-1}$ and still increasing at the maximum concentrations used for ConA or LPS. Maximum count of induced colonies were about 1200, 300 and 50 colonies per well for PHA, ConA and LPS, respectively. These counts varied slightly from trout to trout in different experiments.

When the cultures made in the absence of added mitogens were infected with the IPN virus, they showed cell stimulation as compared to the controls. Table 2 shows the results of several experiments. The lymphocytes present at time 0 in the culture disappeared or were trans-

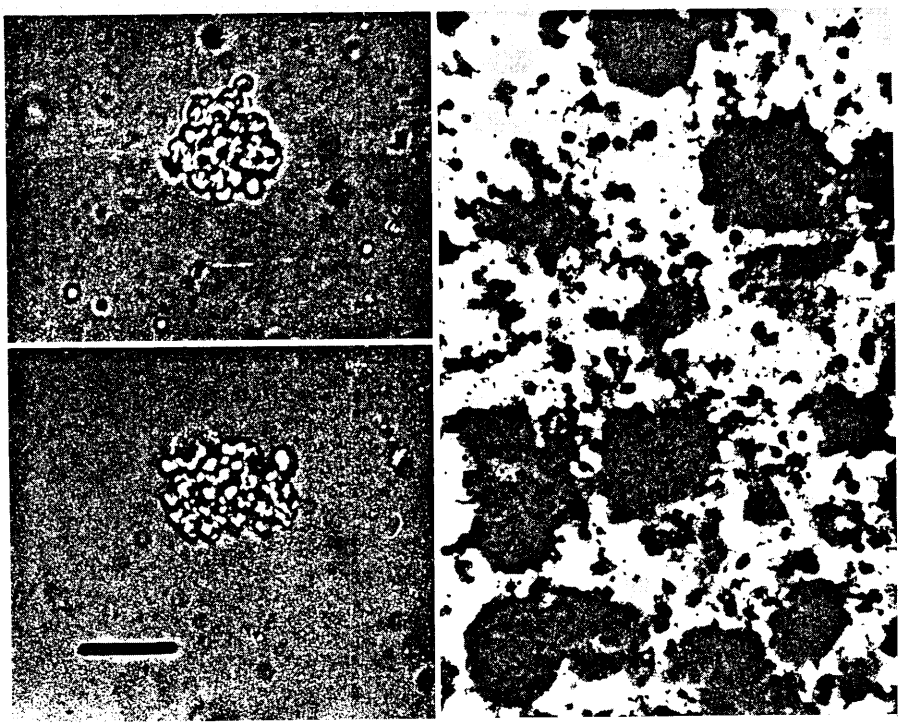

**Figure 1.** PHA-induced colonies in cultures of trout kidney cells. The cultures were made in the presence of 2 $\mu$g of PHA ml$^{-1}$, photographed *in vivo* by phase-contrast (left) or after fixing and staining (right) (bar = 100 $\mu$m).

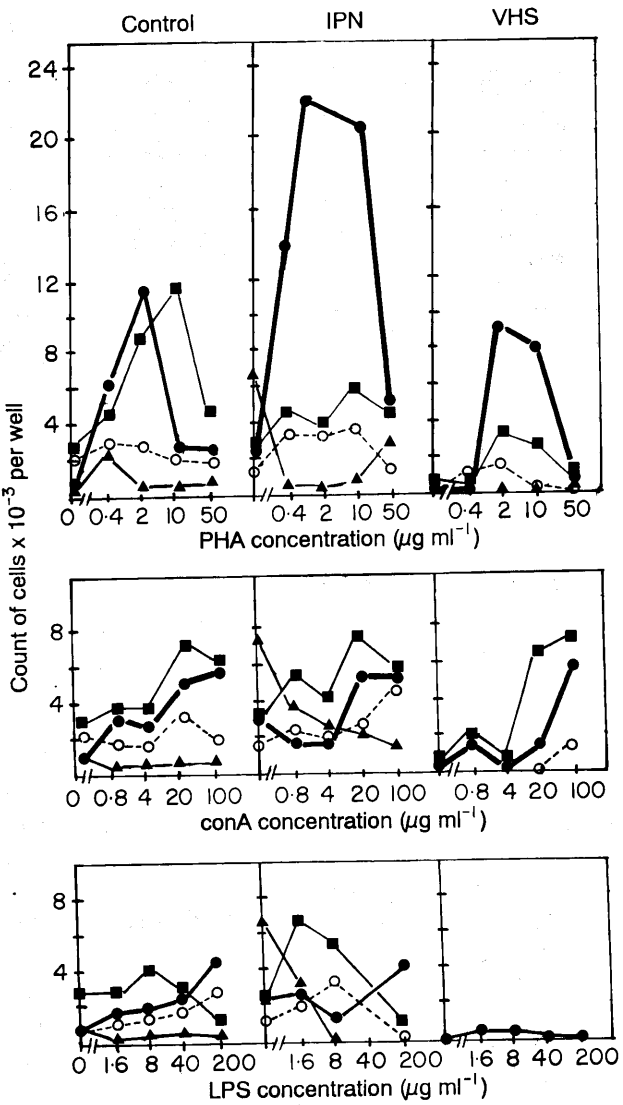


**Figure 2.** Effect of IPN and VHS virus infection on different trout kidney cell types stimulated by PHA, ConA or LPS: (●) eccentric-nuclei cells; (■) multinucleated cells; (▲) large nucleated cells; and (○) adherent cells.

formed to other leucocytes after 2 weeks in both non-infected and infected cultures. Except for lymphocytes, all the rest of the leucocytes were found in the IPN-virus-infected cultures in three-10-fold higher counts than in the non-infected cultures after incubation. In one in four trout, the large nucleated cells formed colonies (250 colonies per well) of between two and six cells per colony. No colonies of any of the cells developed in the rest of the cases studied. When purified IPN was killed by 10 min boiling, and added to the cultures (10 $\mu$g $well^{-1}$), no differences were found with the control cultures (not shown). When the cultures made in the presence of added mitogens were infected with IPN virus a two-fold increase in the count of PHA-stimulated eccentric-nuclei cells was noted (Fig. 2). Other changes were minor. After

**Table 2.** Effect of IPN virus infection on trout kidney cell types, expressed in leucocyte count*

| Type of cells | Time (0) | Time (2 weeks) | |
|---|---|---|---|
| | | Control (S.D.) | IPN (S.D.) |
| Lymphocytes | 11110 (3596) | 0 | 0 |
| Large nucleated | 4742 (1117) | 100 (17) | 1650 (285) |
| Multinucleated | 3825 (2567) | 625 (102) | 1517 (706) |
| Eccentric-nuclei | 3505 (2388) | 287 (270) | 3177 (1513) |
| Adherent | 1296 (776) | 907 (715) | 2970 (1527) |

* Cell types were defined previously (Table 1). Four cultures were made for each individual in each different experiment, and four fields were counted for each experiment. Averages were calculated from the four experiments, standard deviations are given in parenthesis. One trout was used for each experiment. Lymphocytes disappeared after 2 weeks in culture by either death or transformation to other leucocytes.

IPN infection, the count of PHA-induced colonies remained constant, and the count of ConA induced colonies increased by about two-fold (Fig. 3). IPN virus could be recovered in RTG-2 from the supernatant but not from the pellet of the infected clots. The titre varied from $10^2$ to $10^3$ $TCID_{50}$ per well. By immunofluorescence, the leucocytes from infected cultures showed no significant difference when compared with non-infected cultures (not shown).

When the cultures made in the absence of added mitogens were infected with the VHS virus, they always showed a total cell lysis after one week in culture ($n = 6$). When the cultures made in the presence of mitogens were infected with $10^5$ $TCID_{50}\,ml^{-1}$ VHS virus and gassed with $CO_2$ during infection, there was a decrease in the count of both cells (Fig. 2) and colonies (Fig. 3). The extent of this reduction could be controlled by the experimental conditions of the infection. For instance, by using both a higher dosage of virus ($4 \times 10^5$ $TCID_{50}\,ml^{-1}$) and a higher pH (discharging the $CO_2$ after 4–5 days of infection) no surviving cells or colonies appeared in the cultures 7–10 days after infection (not shown). In the cultures stimulated by PHA, the multinucleated cells were reduced by three-fold in contrast to the eccentric-nuclei cells that remained nearly constant in the same cultures.

## Discussion

The present experiments were undertaken to examine the *in vitro* infection with salmonid viruses of trout kidney leucocytes stimulated with mammalian mitogens and to describe the morphology of the possible target cell(s) affected by the infections.

Cultures infected with IPN virus contained, morphologically intact, all the cell types found in non-infected cultures. The leucocyte count tended to be stimulated rather than reduced in the IPN-virus-infected cultures, as compared with either non-infected controls or inactivated IPN-virus-infected cultures (Table 2). Because this enhancement was independent of adding or not adding the mitogens, and the present authors were unable to demonstrate highly fluorescent leucocytes or any virus titre increase after culture, it is suggested that a specific stimulation of leucocytes is taking place in the cultures. This specific stimulation of the leucocytes could be due to a previous exposure to the IPN virus in the farm were these trout came from, in the same way as has been shown for VHS virus (Chilmonczyk 1977). Failure of the

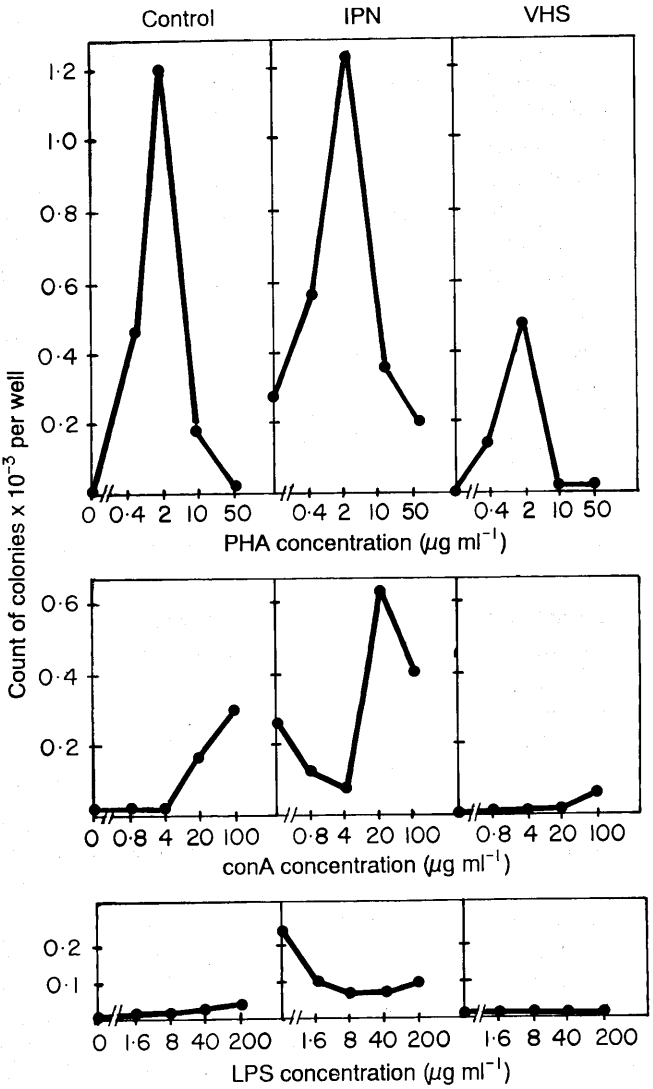


**Figure 3.** Effect of IPN and VHS virus infection on colonies from trout kidney stimulated by PHA, ConA or LPS: (●) eccentric-nuclei cells; (■) multinucleated cells; (▲) large nucleated cells; and (○) adherent cells.

inactivated virus to induce the specific stimulation could be due to the destruction of the required native structures by the heating.

Since an association of the IPN virus with live leucocytes of the trout has previously been demonstrated *in vivo* (Swanson & Gillespie 1982; Yu *et al.* 1982), it could be that infection of trout leucocytes with IPN *in vitro* did not result in the establishment of carrier-cells, as found *in vivo* (Knott & Munro 1986). The results obtained here suggest that trout leucocytes are not a lytic target for the IPN virus.

In contrast to the results obtained with the IPN virus, all the cell types found in the trout leucocytes cultured by the fibrin-clot culture technique (Coll 1990) were affected by the VHS virus infection. No specific cell or colony type was found to be resistant to the infection of the

cultures. VHS viral antigens have been detected in the blood leucocytes of trout surviving experimental infection (Enzmann 1981). These results are in agreement with the idea of trout leucocytes being one of the target cells of the VHS virus. This idea is also indirectly supported by the decreased susceptibility of trout to VHS virus infection after depletion of the trout lymphoid cells by irradiation with $\gamma$-rays (Chilmonczyk & Oui 1988).

Since, on the one hand, inactivated VHS virus induced a specific stimulation of leucocytes in trout surviving natural VHS infection or bath vaccinated with VHS (Chilmonczyk 1977, 1978; de Kinkelin & Le Berre 1977), and on the other, the leucocytes seem to be one of the VHS virus targets (present study), the possibility of using trout kidney cell cultures both for infection with inactivated VHS virus to study immunological memory and for inducing protection to virus challenge *in vitro* is currently under investigation.

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