REPLICATION OF RHABDOVIRUS IN TROUT HEMATOPOIETIC CELLS

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SUMMARY

Viral haemorrhagic septicaemia rhabdovirus (VHSV) infects the hematopoietic cells derived from the pronephritic tissue of the rainbow trout, *Oncorhynchuss mykiss, W.* Primary hematopoietic head-kidney cultures were obtained from whole, isolated macrophages and isolated stromal cells. A non-hematopoietic primary cell culture was stablished from the trout fins and two fish cell lines (RTG-2 from trout and EPC from carp) were used as non-hematopoietic cell controls. Rhabdoviral infection of these cultures resulted in the development of lytic cytopathic effects in all cases. The replication of VHSV was demonstrated by the increase in infectivity as measured by cell line culture assays. The viral titers obtained in all trout primary cell cultures (hematopoietic and nonhematopoietic) were 1000-fold lower than those obtained in the fish cell lines. Possible explanations for these results are discussed, since it might affect both the design of vaccines and the correct diagnosis of fish rhabdoviruses.

KEY WORDS: Rhabdovirus
Hematopoiesis
Trout

INTRODUCTION

Among all the fish diseases and due to its rapid and high mortalities in adult fishes (30-50 p.100 of world annual losses of salmonids in affected areas), the ones caused by rhabdovirus are of the most economic importance. The rhabdoviruses affect to traditionally cultured fish species (trout, salmon), to fish species with a future of culture (sea bass, turbot) and to wild fish species (carp, pike) (Estepa, Coll, 1993).

In salmonids, natural and experimental infections with viral haemorrhagic septicaemia virus (VHSV), one of the most widely distributed rhabdoviruses in Europe, cause severe destruction of the renal hematopoietic tissues (De Kinkelin *et al.,* 1979), its main hematopoietic organ. Necrosis of the interstitial tissue of the kidney is a consistent histopathological sign of VHS (Amlacher *et al.,* 1980; Yasutake, 1968) probably related somehow to the anaemia observed during these rhabdoviral infections (Amend, 1970). However, to date very few studies have focused in the determination of the hematopoietic cellular targets of the rhabdovirus. Since the trout kidney contains cellular precursors of erythrocytes, lymphocytes, granulocytes, macrophages, etc., as well as those fully differentiated cells, we have studied the replication of VHSV in primary cell cultures derived from the trout kid-

Recibido: 28-6-93
Aceptado para su publicación: 24-1-94

ney throughout the years (Diago *et al.*, 1993; Estepa, Coll, 1991a,b; Estepa, 1992; Estepa *et al.*, 1992; Estepa *et al.*, 1993). In this work we review those different studies to examine comparatively all the gathered data. We have found a consistent 1000-fold decrease in the rhabdoviral titers obtained in primary trout culture as compared to those obtained in fish cell lines. Reasons for this discrepancy and its possible importance to vaccine design and correct diagnosis are discussed.

MATERIAL AND METHODS

Rainbow trout, *Onchorynchus mykiss,* Walbaum

Adult trout (180-250 g), *Onchorynchus mykiss W.*, were purchased from commercial fish farms after tests indicated they were free of infectious pancreatic necrosis virus. Fish were maintained in pathogen-free water in closed system aquaria at 15° ± 1 °C.

Culture of pronephric cells

Culture medium consisted of RPMI-1640 containing 20 mM HEPES buffer (Gibco, Grand Island, NY, USA), supplemented with L-glutamine (5 mM), sodium pyruvate (2.5 mM), 2 β-mercaptoethanol (50 μM), gentamicin (100 μg/ml), fungizone (2 μg/ml), 10 p. 100 fetal calf serum (FCS) and 0.5 p. 100 rainbow trout pooled sera.

Primary cell cultures were obtained from explants of head kidney (pronephros), as previously described (Estepa *et al.*, 1991, 1992). Briefly, trout were anaesthetized with 0.05 p. 100 of tricaine methanesulfonate (MS-222, Sandoz) bled by the caudal vein and the pronephros removed under sterile conditions. Tissue fragments were washed in PBS (0.05 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) and then passed repeteadly throughout a syringe. Cell suspensions were centrifuged at 300 X g at 4 °C for 10 min and resuspended in 5 ml of culture medium.

Whole kidney hematopoietic cells were cultured at 2×10^5 round cells/ml, at 20 °C after gassing with 5 p. 100 CO_2 in air. To isolate their macrophage population, 3×10^6 round cells from the whole head kidney were incubated in 25 cm^2 plastic flasks in 5 ml of the cell cultured medium at 20 °C. After ten days, the medium was changed to eliminate the non-adherent cell population and the macrophages were kept at 14 °C until used for the experiments. The yield was about 1×10^6 macrophages per flask (Estepa *et al.*, 1992)

Pronephric stromal cells were obtained from adherent cell populations subcultured 9 times (9-month subcultures, 9 passages, one passage per month). This passage number was chosen to have enough cells to repeat experiments. They were capable of undergoing spontaneous hematopoiesis as described (Diago, 1990; Diago *et al.*, 1991).

To isolate fin cells, after anaesthesizing the trout, one of the ventral fins was cut and removed from the trout. After the surgical operation the wounds of the trout were desinfected with ethanol and the trout were put back into their closed system aquaria with the addition of 250 μg fungizone/ml and 50 μg of gentamicin/ml. Under these conditions trout survival was 100 p. 100. The fin fragment was further cut into pieces in a Petri-dish and incubated in 0.5 p. 100 Trypsin-

10 mM EDTA in RPMI-1640 for 5 to 10 min. Clumps of cells were dissociated by passing the suspension through a Pasteur pipette. The cell suspension was centrifuged at 1000 x g for 10 min, resuspended in cold cell culture medium and cultured at 14 °C in 25 cm^2 cell culture flasks.

Rhabdoviral infection of the cell cultures

The strain of rhabdovirus used was the VHSV 0.7.71, gift of Dr. de Kinkelin (INRA, Jouy en Josas, France) isolated from rainbow trout. The virus was propagated by infection with low m.o.i. (0.0001 virus per cell) of a fibroblastic trout cell line, the rainbow trout gonad (RTG-2) and in an epithelial carp cell line, the epithelioma papullosum cyprini (EPC). After the infected EPC cells exhibited complete cytopatic effect, the supernatants were harvested, their cellular debris centrifuged away, their VHSV titrated (Basurco, 1990; Basurco, Coll, 1989; Estepa, Coll, 1992) and then used to infect the trout primary cell cultures (Estepa, Coll, 1992; Estepa *et al.*, 1991).

Cells from the trout were resuspended in culture medium at a density of about 10^4-10^5 cells/ml. Cultures were infected with VHSV at different multiplicities of infection (m.o.i.), and incubated at 14 °C (the optimal temperature for the in vitro VHSV infection) in the absence of CO_2 gassing for about ten days or until complete cytopathic effect. For these experiments, low m.o.i. were used to minimize the possible inhibition of infection due to the potential presence of interfering defective particles in the inoculum. Control non-infected cell cultures were incubated at 14 °C during the same time.

Titration of virus

To assay the VHSV recovered after infection of the trout cell cultures, the supernatants were centrifuged at 5000 X g for 10 min and titrated in EPC monolayers by the $TCID_{50}$ method as described (Basurco, 1990). Immediately prior to the titration, VHSV was also added to the control cultures, to allow a distinction to be made between viral replication and survival.

To corroborate the infectious viral titers, free nucleoprotein N viral antigen (the protein of the nucleocapsid of VHSV) was assayed by an enzyme-linked immunosorbent assay (ELISA) based in monoclonal antibodies (MAbs) as described (Sanz, Coll, 1992). The 2 MAbs used were the anti-N protein 2C9 (conjugated to horseradish peroxidase) and the anti-N protein 2D5 (to coat the plates). The supernatants from infected cell cultures were tested by 2-fold dilutions in duplicates. Titer was defined as the reciprocal of the dilution to give a 492 nm absorbance of 0.4 (0.25 absorbance units above the cut-off value from the background of 0.15).

Flow cytometry

To further corroborate the VHSV infection, the presence of VHSV antigens in the cells was analysed by flow cytometry, following the method described before (Estepa *et al.*, 1991). Five day post-infected cells were, resuspended in PBS, containing 1 p. 100 bovine serum albumin, and 0.1 p. 100 sodium azide (PBS-BSA-NaN_3). The detached cells were centrifuged at 300 X g for 10 min

and the pellet gently resuspended in PBS-BSA-NaN_3 containing 100-fold diluted mouse ascites with anti-VHSV polyclonal antibodies. After 1 hour of incubation at 20 °C with occasional agitation, the cell suspensions were washed in PBS-BSA-NaN_3, resuspended in the same buffer containing 700-fold diluted rabbit anti-mouse IgG-FITC conjugate (Nordic, Tilburg, The Netherlands), and incubated during 30 min at 20 °C. Cells were washed twice and fixed by resuspending them in 0.3 p. 100 paraformaldehyde in PBS, either in the presence (stromal cells) or absence of 0.02 p. 100 Triton X-100 (rest of the cells). The same day of harvest and staining, 5000 cells were analysed by flow cytometry in a Beckton Dickinson (San Jose, California) FACScan apparatus, using the program LYSYS II (v. 1.0). Green fluorescence was measured at 514-545 nm (FL1 filter).

RESULTS

That the trout kidney cell cultures inoculated with VHSV, were susceptible to this rhabdovirus, was indicated by the occurrence of cytopathic effects a few days later. Parallel control uninfected cultures maintained at 14 °C did not show any changes. The rhabdoviral infections resulted in the detachment of scattered cells (in macrophage, stromal or fin cell cultures) after 3-5 days. At seven days postinfection or later depending on the m.o.i., the cultures showed small areas devoid of cells. Cultures were totally lysed by more than ten days postinfection, independently of the initial m.o.i. used (ranging from 0.0001 to 0.5). All the kidney cell types (Coll, 1990) in the whole kidney cell culture infected with VHSV were either dead or had low survival rates. Most of the cells in these cultures were also completely lysed, as above.

VHSV were released to the supernatant of the infected cell cultures, as demonstrated by the analysis of the supernatants by the $TCID_{50}$ method (Table 1). The final VHSV titer obtained in the supernatant for every 200,000 infected cells was 6,400, 2,500 and 2,500 $TCID_{50}$/ml for whole kidney, isolated macrophages and isolated stromal cells, respectively. Non-hematopoietic (fin cells) trout primary cell cultures produced a VHSV titer of 12,500 $TCID_{50}$/ml. In contrast with the trout primary cultures, VHSV infection of fish cell lines, either RTG-2 and/or EPC produced a VHSV titer $\geq 10^7$ $TCID_{50}$/ml. These results were confirmed by detection of the nucleoprotein N by ELISA. Increments of the initial N titer were found between 2-fold (whole kidney) and 20-fold (macrophages, stroma and fin cells) whereas at least a 100-fold increment in their titer was obtained in supernatants from VHSV infected RTG-2 or EPC (not shown).

The percentage of anti-VHSV cells that have positive levels of immunofluorescence varied from 20 p. 100 (in stromal cells, intracellular antigens) to 50 p. 100 (in macrophages, plasma membrane antigens) (Fig. 1).

DISCUSSION

Trout cells capable of in vitro hematopoiesis (Estepa, Coll, 1992; Diago, 1990; Diago *et al.*, 1991) isolated from rainbow trout pronephros, were susceptible to viral haemorrhagic septicaemia virus (VHSV). The immunofluorescence

TABLA 1

VHSV REPLICATION ON TROUT HEMATOPOIETIC CELLS

Replicación del VSHV en células hematopoyéticas de trucha

Cell type	m.o.i.	$TCID_{50}$/ml	$TCID_{50}$/cell	Reference
WK	0,008	$6,4 \times 10^3$	0,032	Estepa *et al.*, 1991
IM	0,0001	$2,5 \times 10^3$	0,012	Estepa *et al.*, 1992
IS	0,5	$2,5 \times 10^3$	0,012	Diago *et al.*, 1993
CF	0,02	$1,25 \times 10^3$	0,062	Estepa *et al.*, 1993
RTG-2	0,0001-1	$\geq 10^7$	50	Basurco, 1990
EPC	0,0001-1	$\geq 10^7$	50	Basurco, 1990

Cell cultures were infected with VHSV at low multiplicity of infection (m.o.i.) and supernatants after ten days at 14 °C were titrated on EPC monolayers. Results expressed as $TCID_{50}$/ml/200,000 cells. WK, whole kidney; IM, isolates macrophage; IS, isolated stroma; FC, fin cells. RTG-2 and EPC can be infected with VHSV over a wide range of m.o.i. without affecting viral yield.

Los cultivos celulares se infectaron con VHSV a baja multiplicidad de infección (m.o.i.) y los sobrenadantes después de diez días a 14 °C se titularon en monocapas de EPC y los resultados se expresan en $DICT_{50}$/ml/200,000 células. WK, riñón entero; IM, macrófagos aislados; IS, stroma aislado; CF, células de aleta. Las células RTG-2 y EPC se pueden infectar con un rango amplio de m.o.i. sin afectar el rendimiento viral.

staining with anti-VHSV PAbs, the release of VHSV particles to the supernatant as demonstrated by the EPC cell culture assay and the increase of the VHSV nucleoprotein N antigen by ELISA, indicate that VHSV replicate in all the hematopoietic cell cultures studied. These *in vitro* results help to explain the numerous *in vivo* observations reporting the destruction of the renal interstitial tissue during VHS (Amlacher *et al.*, 1980; De Kinkelin *et al.*, 1979 Yasutake, 1978; Yasutake, Rasmussen, 1968) or infectious hematopoietic necrosis, IHN (Amend, Chambers, 1970; Yasutake, 1978), two rhabdoviral-caused fish diseases. Other *in vivo* studies involving the detection of rhabdoviral antigens (Enzman, 1981) and the depletion of lymphocytes by irradiation (Chilmonczyk, Oui, 1988), have also demonstrated that some trout leucocytes can be infected by rhabdovirus.

Whether hematopoietic kidney stromal cell cultures (Diago *et al.*, 1993), kidney total leucocytes (Estepa, Coll, 1991a), isolated kidney macrophages (Estepa *et al.*, 1992) or fin cells (Estepa *et al.*, 1993) were infected with VHSV, rhabdovirus yields were similar (2.5, 6.4, 2.5, and 12.5×10^3 $TCID_{50}$ per ml per 200,000 cells, respectively). In contrast, VHSV yields of 10^7-10^8 $TCID_{50}$/ml per 200,000 cells were obtained by using the RTG-2 or the EPC cell line (Basurco, 1990). The production of VHSV goes from 0.01-0.06 $TCID_{50}$/cell in the trout hematopoietic primary cell cultures to 50 $TCID_{50}$/cell in the fish cell lines. What is the reason(s) for this 3-order of magnitud difference? One first possible explanation would be that not all the cells present in the primary cultures are lytic targets of the rhabdovirus. However, since all the cells are lysed as shown by visual inspection, could it be possible that the cell lysis would be due to some toxic component released by the infected cells? That this last explanation is not probably the case, is suggested by the high percentage of VHSV infected cells that was estimated by cytofluorometry (between 20 to 50 p. 100 depending on the culture). Other possible explanation could be that not enough infective rhabdoviruses are released. However the ELISA results (which detect the N antigenic component of the rhabdovi-

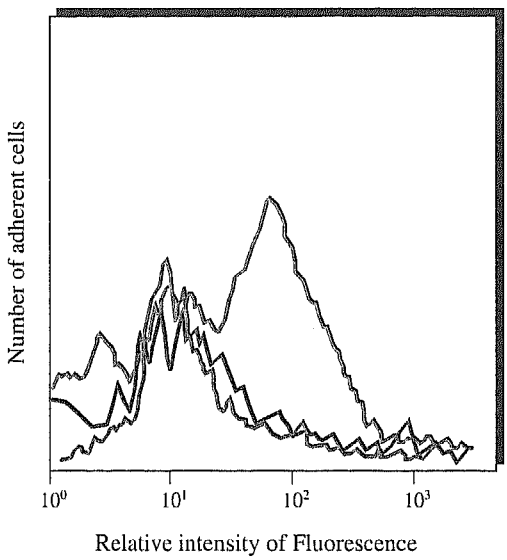

Fig. 1.–Membrane fluorescence at 515-545 nm measured by flow cytometry of VHSV-infected macrophages stained with anti-VHSV polyclonal antibody (PAb) raised in mice. Relative fluorescence intensity (X axis) versus number of cells (Y axis), red VHSV-infected macrophages; green non-infected macrophages, blue no PAb.
Fluorescencia de membrana a 515-545 nm medida por citoflurometría de flujo de macrófagos infectados con VSHV y teñidos con anticuerpos policlonales (AcP) de ratón. Intensidad relativa de fluorescencia (X) respecto al número de células (Y), rojo macrófagos infectados con VSHV, verde macrófagos no infectados, azul no AcP.

rus independently of if it is infective or not) also detect a similar though smaller difference in titer (Diago *et al.*, 1993; Estepa *et al.*, 1991, 1992). A higher production of non-infective but interfering defective particles in the primary trout cell culture compared to the fish cell lines could offer an alternative possible explanation. If most of the rhabdovirus released during the VHSV infection of the primary trout cell cultures are defective, this would cause not only a small decrease in the viral antigen titer but also a high decrease in the rhabdoviral titer because of the interfering properties of the defective rhabdoviral particles. The production of defective particles is a well known phenomena in all rhabdoviruses and it has been described in fish rhabdovirus (Amend, 1970; Amlacher *et al.*, 1980; De Kinkelin *et al.*, 1979; Basurco, 1990). Large scale experiments should be performed to assay for the production of defective interfering particles in these primary cell cultures. If proven, this fact could have importance in our knowledge of the in vivo rhabdoviral infection for the design of effective vaccines and also will affect its diagnosis. It this last respect, it is well known, in fish viral diagnosis, the difficulty in demonstrating the presence of rhabdoviruses from VHSV dead fish at low dilutions of the organ homogenates because of the abundance of defective interfering particles (false negatives). Further studies should be made to explore this and/or other possibilities because of their possible importance to both vaccines and diagnostics.

ACKNOWLEDGEMENTS

We appreciated the technical assistance of J. Coll Perez in typing. This work was supported by Research Grants AGF92-0059 from the Comision Interministerial de Ciencia y Tecnología (CICYT), Spain and CT920036 from the AIR program of the Comission of the European Communities (CEE).

RESUMEN

Replicación de rabdovirus en células hematopoyéticas de trucha

El rabdovirus de la septicemia hemorrágica viral (VSHV) de la trucha, infecta las células hematopoyéticas derivadas del tejido pronefrítico de la trucha Arco Iris, *Oncorhynchuss mykiss*, *W*. Los cultivos primarios de células hematopoyéticas del riñón anterior se obtuvieron de riñón entero, de macrófagos aislados y de células de estroma aisladas. Un cultivo primario de aletas de trucha y dos líneas celulares de peces (RTG-2 de trucha y EPC de carpa) se utilizaron como controles de células no hematopoyéticas. La infección vírica de estos cultivos produjo efectos citopáticos líticos. La replicación del VSHV se demostró por un aumento de su infectividad mediante ensayos en líneas celulares. Los títulos virales obtenidos en todos los cultivos primarios de trucha estudiados (hematopoyéticos y no hematopoyéticos) fueron 1.000 veces menores que los obtenidos en las líneas celulares. Estos resultados pueden tener repercusiones tanto en el diseño de vacunas como en el diagnóstico de los rhabdovirus en peces.

PALABRAS CLAVE: Rabdovirus
Hematopoyesis
Trucha

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