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The *In Vitro* Infection of the Hematopoietic Stroma of Trout Kidney by Hemorrhagic Septicemia Rhabdovirus

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ABSTRACT

Viral hemorrhagic septicaemia virus (VHSV) infected the hematopoietic stromal cells (7,8) derived from pronephritic tissue of the rainbow trout, *Oncorhynchuss mykiss*, W., at their ninth passage *in vitro*. Viral infection resulted in the development of lytic cytopathic effects on confluent *in vitro* tridimensional network stromal cell cultures. Replication of VHSV in the stromal cell cultures was demonstrated by the increase in infectivity by epithelioma papulosum cyprini (EPC) cell culture assays and by the increase of the nucleoprotein antigen of VHSV by ELISA. By using anti-VHSV monoclonal antibodies (MAbs), flow cytometry studies demonstrated that only the infected stromal cells contained cytoplasmic viral antigens. The lytic infection of trout hematopoietic stromal cells *in vitro* could be relevant to the hemorrhagic pathology seen in the kidney of fish infected with VHSV.

INTRODUCTION

In salmonids, natural and experimental infections with viral hemorrhagic septicemia virus (VHSV) cause severe destruction of the renal hematopoietic tissues (6). Necrosis of the interstitial tissue of the kidney is a consistent histopathological sign of VHS (2,21) probably related to the anemia observed during these viral infections (6).

Studies involving the detection of viral antigens (10), the depletion of lymphocytes by irradiation (5), and the *in vitro* infection of leukocyte cultures (12,13) have demonstrated that trout leukocytes can be infected by VHSV. VHSV replication *in vitro* has been demonstrated in kidney leukocytes (12), mitogen-induced leukocyte colonies (13), and isolated macrophages (16). However, the possible susceptibility of the hematopoietic stromal cells, or their possible role as loci for VHSV replication has not been described yet.

The subcultures of pronephric stromal cells used in this study have been recently described and their cells identified as stromal and capable of undergoing spontaneous hematopoiesis under special conditions (7,8). Noninfected subcultures of stromal cells consisted of tridimensional networks of two main adherent cell types. One of the types, formed stellate, nonphagocytic, fibroblastic-like cells, resembling structural and histochem-

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ically the reticular cells forming the network of the hematopoietic tissue (7,8). The other type consisted in large, rounded epitheloid cells, showing numerous vesicles in the cytoplasm and exhibiting characteristics of the sinusoidal cells, which line the renal blood sinusoids *in vivo*. These cells and endothelial cells are suspected targets of rhabdovirus, their destruction probably contributing to the hemorrhages characteristic of the disease.

We demonstrate here that the stromal cell populations, isolated by subculturing primary long-term cultures from trout pronephros, are susceptible to infection by VHSV. We further demonstrate the productive replication of VHSV in these cells.

MATERIALS AND METHODS

Rainbow trout, *Onchorynchus mykiss*, Walbaum. Adult trout (180–250 g), *Onchorynchus mykiss* W., were purchased from a commercial fish farm (Los Leoneses, Castrillo del Porma, León). After tests indicated they were free of infectious pancreatic necrosis, fish were maintained at the aquarium of the Departamento de Biología Celular y Anatomía (León), in 400-liter tanks supplied with running dechlorinated, pathogen-free water at 15 ± 1°C.

Culture of pronephric stromal cells. Culture medium consisted of RPMI-1640 containing 25 mM HEPES buffer (Gibco, Grand Island, NY), supplemented, in addition to normal constituents, with L-glutamine (2–5 mM), sodium pyruvate (2.5 mM), the four nucleosides (25 mM each), 2-mercaptoethanol (5 mM), gentamycin (100 μg/ml), fungizone (2 μg/ml), 10% FCS, and 5% rainbow trout pooled sera. The pH of the medium was adjusted to 7.4 and the osmolarity to 295 mOsmol/kg.

Primary stromal cell cultures were obtained from explants of pronephros, as previously described (7). Briefly, trout (180–250 g) were anesthetized with 0.05% of tricaine methanesulfonate (MS-222, Sandoz) bled by the caudal vein and the head kidney (pronephros) removed in sterile conditions. Organ fragments of approximately 1 mm^3 were washed in PBS (0.05 M sodium phosphate, 0.15 sodium chloride, pH 7.2) and then incubated in 24-well culture plates (Costar, Cambridge, MA) with 2 ml of culture medium at 18°C in a humidified air atmosphere. Cell monolayers were detached by 2 min exposure to 0.05% trypsin, 0.02% EDTA in a 0.85 g/liter NaCl aqueous solution at 18°C, for subculture. Cell suspensions were centrifuged at 300 *g* at 4°C for 10 min, resuspended in 5 ml of culture medium at 1×10^4 cells/ml, and plated onto 25-cm^2 flasks (Costar). Subcultures were incubated as indicated above. Pronephric stromal cells used in these experiments were obtained from 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 previously (7,8).

Viral infection of stroma cultures. The strain of virus used was the VHSV 0.7.71, a gift of Dr. de Kinkelin (INRA, Jouy en Josas, France), isolated from rainbow trout. The virus was cultured in an epithelial carp cell line, the epithelioma papillosum cyprini cells (EPC). After the infected EPC cells exhibited complete cytopathic effect the supernatants were harvested, their cellular debris centrifuged away, their VHSV titrated (3,14), and then used to infect the stromal cultures (12,13,16).

Cultured stromal cells were resuspended in culture medium at a density of 7.5×10^4 cells/ml, and 5-ml aliquots were plated in 25-cm^2 flasks. After 4 days incubation at 18°C, cultures containing 10^6 cells/flask were infected with VHSV at different multiplicities of infection (moi), and incubated at 14°C (the optimal temperature for the *in vitro* VHSV infection) for several days depending on the experiment. Control noninfected stromal cell cultures were incubated at 14°C during the same time.

Titration of virus. To titrate the VHSV recovered after infection of the stromal cell cultures, the supernatants were centrifuged at 5000 *g* for 10 min and titrated in EPC monolayers by the $TCID_{50}$ method as described before (3,14). Immediately prior to titration, VHSV was added to the control cultures, to allow a distinction to be made between viral replication and virus survival.

To corroborate the infectious virus titers, free nucleoprotein N viral antigen (the protein of the nucleocapsid of VHSV) was titrated by the enzyme-linked immunosorbent assay (ELISA). The centrifuged supernatants from virus-infected stromal cultures were tested for the presence of the VHSV nucleoprotein N by the a monoclonal antibodies (MAbs)-based ELISA sandwich, as previously described (18). The 2 MAbs used were

anti-N protein 2C9 (conjugated to horseradish peroxidase) and 2D5 (to coat the plates). The supernatants from infected 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 stromal cells was analyzed by flow cytometry, following the method described before (16). Five day postinfected cultures of stromal cells were detached from the flask surface by exposure to trypsin-EDTA, and resuspended in PBS pH 7.4, containing 1% bovine serum albumin, 0.1% sodium azide, and 0.01% Triton X-100 (PBS-BSA-NaN_3). The detached cells were centrifuged at 300 *g* for 10 min and the pellet gently resuspended in PBS-BSA-NaN_3 containing 100-fold diluted mouse ascites with an IgG_1 anti-VHSV glycoprotein MAb (1H10), an IgG_{2a} anti-VHSV nucleoprotein MAb (2C9) (15), or an IgG_1 anti-trout IgM (1A6) (19). MAb anti-trout IgM (1A6) (an MAb exhibiting no binding to noninfected stromal cells) was chosen as a control against infected cells nonspecifically binding mouse IgG. After 1 hr 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% paraformaldehyde in PBS, either in the presence or absence of 0.02% Triton X-100. The same day of harvest and staining, 5000 cells were analyzed by flow cytometry in a Beckton Dickinson (San Jose, CA) FACScan apparatus, using the program LYSYS II (v. 1.0). Green fluorescence was measured at 514–545 nm (FL1 filter).

RESULTS

That the stromal subcultures inoculated with VHSV were susceptible to this virus was indicated by the occurrence of cytopathic effects a few days later (Fig. 1A). Parallel control uninfected cultures maintained at 14°C did not show any changes. The viral infections resulted in the detachment of scattered cells after 3–5 days. At 7 days postinfection or later depending on the moi, the cultures showed small areas devoid of cells. A more general cytopathic effect was observed later on, when most of the stromal cells rounded-up after 10 days (Fig. 1B). Cultures were totally lysed by 3 weeks postinfection independently of the initial moi used (ranging from 0.001 to 0.5).

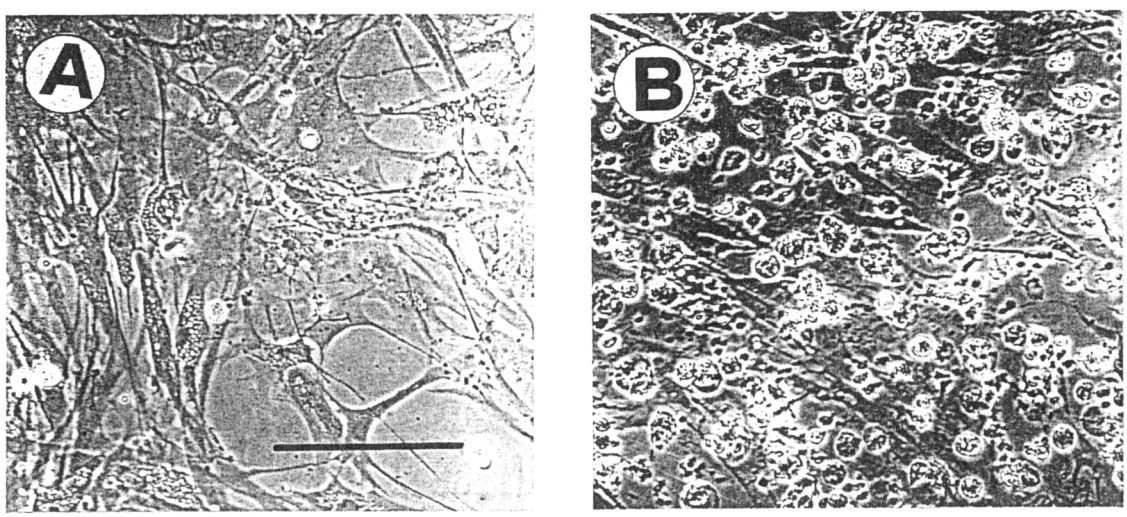

FIG. 1. (A) Phase contrast micrograph of 7-day VHSV postinfected pronephric stromal cells from 9 months of culture (9 passages). (B) Phase contrast micrograph of the same VHSV infected cultures as above 10 days postinfection. The horizontal black bar is 100 μm (photographs were taken at ×330).

VHSV were released to the supernatant of the infected stromal cell cultures, as demonstrated by the analysis of the supernatants by the $TCID_{50}$ and/or the ELISA methods. A 25- or 20-fold increment of their titer was obtained 1 week after infection at a moi of 0.5 virus/cell by $TCID_{50}$ or by ELISA, respectively (Table 1).

To further corroborate the infection of the stroma cells, the presence of VHSV antigens in the infected cells was estimated by flow cytometry with the help of anti-VHSV MAbs. Due to the need of trypsinization of the cell monolayers for the flow cytometry analysis, Triton X-100 had to be included in the buffers, otherwise no differences in the intensity of fluorescence could be found between noninfected and VHSV-infected stromal cells (not shown). The average cellular size was not different in the trypsinized stromal cell populations between noninfected and VHSV-infected cell cultures 5 days after infection (not shown). However, when stained with anti-VHSV MAbs anti-G (1H10) or anti-N (2C9), the VHSV-infected stromal cell cultures showed a fluorescence intensity 2- to 3-fold higher than the corresponding noninfected controls or than the cultures stained with a control MAb (anti-trout IgM, 1A6) (Fig. 2).

DISCUSSION

Stromal cells capable of *in vitro* hematopoiesis (7,8) isolated by the subculture of explants from rainbow trout pronephros were susceptible to viral hemorrhagic septicemia virus (VHSV). Although VHSV (6) and the related fish rhabdovirus, the infectious hematopoietic necrosis (IHNV) can replicate in numerous fish cell lines or in trout leukocytes (12,13), there are no *in vitro* studies using stromal cell cultures from the hematopoietic tissue, which histologically appears to be one of the main *in vivo* targets for both rhabdoviruses (6). This study demonstrates that cultured stromal cells from the pronephros are targets for VHSV. Parallel results were also obtained by using IHNV (Cedar Strain) (not shown). Whether hematopoietic kidney stromal cell cultures (this work), kidney leukocytes (12), isolated kidney macrophages (16), or fin cells (17) were infected with VHSV, virus yields were similar (2.5, 6.4, 2.5, and 12.5 $\times$ 10^3 $TCID_{50}$ per ml per 200,000 cells, respectively). In contrast, virus yields of 10^7–10^8 $TCID_{50}$/ml per 200,000 cells were, however, obtained by using the EPC cell line (3).

The stromal cytoplasmic staining with the anti-G and anti-N MAbs anti-VHSV (Fig. 2), the release of VHSV particles to the supernatant as demonstrated by the EPC cell culture assay (Table 1) and the increase of the VHSV nucleoprotein N antigen as demonstrated by ELISA (Table 1) indicate that VHSV replicate in the stromal cell cultures. These *in vitro* results help to explain the *in vivo* observations reporting the destruction of the renal interstitial tissue during VHS (2,6,21) or IHN (1,20) diseases.

The subcultures of the pronephric stromal cells resemble the *in vivo* hematopoietic microenvironment, and they can support hematopoiesis (7,8). The lysis of stromal cell types by an *in vivo* rhabdovirus infection will, thus, produce disturbances on the tridimensional stromal microenvironment. The presence of an intact microenvironment is a requisite for *in vivo* hematopoiesis in higher vertebrates (9) and probably in fish (4,22).

TABLE 1. VHSV REPLICATION (EPC CULTURE) AND INCREASE IN NUCLEOPROTEIN N ANTIGEN (ELISA) IN VHSV-INFECTED TROUT STROMAL CELL CULTURES[a]

		VHSV	
Method	*Time of virus addition*	*$TCID_{50}$/ml*	*ELISA titer*
EPC monolayers	After incubation	100000	—
	Before incubation	2500000	—
ELISA	After incubation	—	50
	Before incubation	—	1000

[a]Cell cultures infected with virus and incubated for 1 week at 14°C. VHSV (moi of 0.5 $TCID_{50}$ VHSV per cell) were added before the incubation or after the incubation. Results are expressed as $TCID_{50}$/ml after titration on EPC monolayers (3) or as the inverse of the dilution to obtain an absorbance of 0.4 after estimation by ELISA (18).

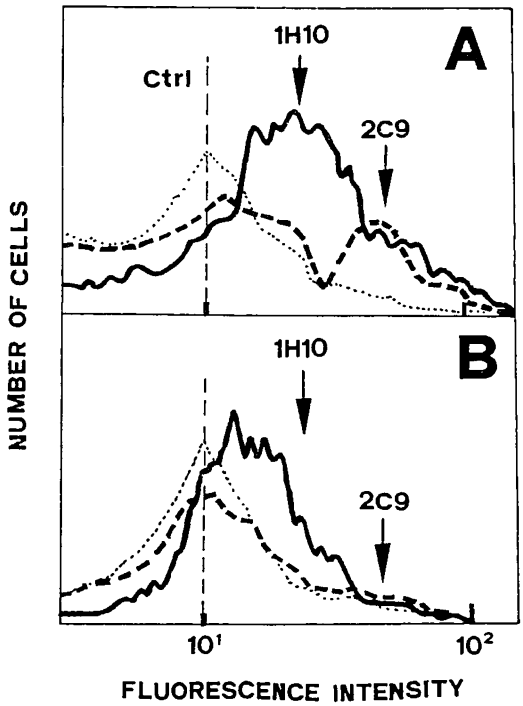

FIG. 2. Fluorescence at 515–545 nm measured by flow cytometry of MAb-stained VHSV-infected stromal cell monolayers. *X*-axis, fluorescence intensity; *Y*-axis, number of cells. The same number of cells has been represented in A and B. (A) Infected cells stained with MAbs anti-VHSV, anti-G (1H10), anti-N (2C9), or control MAb. (B) Noninfected cells completely devoid of virus and stained with the same MAbs. (——) MAb anti-G 1H10; (– – –), MAb anti-N 2C9; (· · · · ·), Ctrl, control anti-trout IgM MAb 1A6. The vertical dashed line shows the anti-trout IgM (19) fluorescence background shown by most of the cells stained with this control MAb.

Therefore, the rhabdovirus infection of the hematopoietic tissues *in vivo* may result in an altered hematopoiesis, even in mild (low virulence) or chronic viral infection conditions.

As stromal cells have a long life span and leukocytes are targets for VHSV (12,13) (and probably also for IHNV) (1), the VHSV-infected stromal cells could hypothetically deliver the virus to their differentiating leukocytes (4,7,8) during *in vivo* chronic infections (11). However, the lytic nature of the *in vitro* infected stromal cells suggests that rhabdovirus-infected stromal cells do not constitute viral reservoirs for recurrent infection of the trout hematopoietic cells. Furthermore, VHSV could not be isolated or detected by cell culture or by the polymerase chain reaction, respectively, in stromal cultures established from VHSV-resistant trout (data not shown).

Finally, it is possible that stromal cells express viral epitopes on their cell surface and not only intracellulary (as demonstrated in this work), as it happens with infected macrophages (16). In that case, infected stromal cells might act as antigen-presenting cells to generate an immune response to the rhabdoviruses. However, no differences in the intensity of membrane fluorescence could be found between control- and infected-stromal cell cultures (not shown), although this might be due to the need of trypsinization (which would destroy the membrane epitopes) to obtain the stromal cell suspensions. Further studies by removing the adherent cells without the use of trypsin (for instance, by increasing EDTA in the absence of divalent cations) should resolve this question.

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