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Short communication

Induced fusion of VHSV persistently infected fish cells

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Viral haemorrhagic septicaemia virus (VHSV) is a rhabdovirus which causes an acute disease with an important economic impact in trout culture in Europe (Jensen 1965; Leong, Bootland, Anderson, Chiou, Drolet, Kim, Lorz, Mourich, Ormonde, Perez & Trobridge 1995). *In vivo*, in surviving fish, virus-carrier states might become established as reported for the similar fish rhabdovirus, infectious haematopoietic necrosis virus (IHNV) (Drolet, Chiou, Heidel & Leong 1995). *In vitro*, cells persistently infected with mammalian rhabdoviruses such as rabies (Webster, Charlton & Casey 1989) and vesicular stomatitis virus (Ahmed & Lyles 1997; Jordan & Youngner 1987) have been described. These have also been described for fish rhabdoviruses like IHNV (Drolet *et al.* 1995; Engelking & Leong 1981), but not for VHSV. *In vitro* persistence of rhabdoviral infection was apparently mediated by several factors which included development of temperature-sensitive viral mutants, small plaque viral mutants and defective interfering viral particles (Engelking & Leong 1981).

Cell to cell fusion of *epithelioma papulosum cyprini* (EPC) cells infected with VHSV was dependent on pH (5.6) and temperature (14 °C) (Estepa & Coll 1996, 1997). Similar results were reported in insect cells either infected with VHSV (Lorenzen & Olesen 1995) or transfected with plasmids expressing the G protein (Lecocq-Xhonneux, Thiry, Dheur, Rossius, Vanderheijden, Martial & deKinkelin 1994).

In this study, the present authors have isolated cell lines from VHSV-infected EPC cells which survived virus infection. The cells showed viral protein expression. This is the first report of the existence of persistently VHSV infected cells. Some of these clones could be used as a model to study VHSV-induced cell fusion.

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Viral haemorrhagic septicaemia virus-07.71, isolated in France (LeBerre, deKinkelin & Metzger 1977) from rainbow trout, *Oncorhynchus mykiss* (Walbaum), was grown and assayed for infectivity in EPC cells (Fijan, Sulimanovic, Bearzotti, Muzinic, Zwillenberg, Chilmonczyk, Vautherot & deKinkelin 1983).

To obtain anti-VHSV polyclonal antibodies, each of three female BALB/c mice were first injected with 20 μg of PEG-concentrated VHSV (Basurco, Sanz, Marcotegui & Coll 1991) in Freund's complete adjuvant and then four monthly injections in Freund's incomplete adjuvant were performed. To obtain ≈ 40 mL of pooled diluted ascites, the mice were intraperitoneally injected with 2×16^6 viable myeloma X63/Ag8653 cells per mouse (Coll 1989). Immunoglobulins were then purified from the pooled ascites by affinity chromatography over a prot A-Sepharose column (Pharmacia, Uppsala, Sweden) (Fernandez-Alonso, Lorenzo, Perez, Bullido, Estepa, Lorenzen & Coll 1998). Anti-peptide polyclonal antibodies were raised as described previously (Estepa & Coll 1997).

For assay of viral antigen expression, the persistently VHSV-infected EPC monolayers were fixed for 10 min in cold methanol and dried. To detect VHSV antigens, monoclonal (Sanz & Coll 1992) and polyclonal antibodies 500-fold diluted in ELISA dilution buffer (130 mM NaCl, 2 mM KCl, 8 mM Na_2 HPO_4, 1.4 mM KH_2PO4, 0.24 mM merthiolate, 5 g L^{-1} bovine serum albumin, 0.3%

rabbit serum, 0.5 g L^{-1} Tween 20 and 50 mg L^{-1} phenol red, pH 6.8) were added to the wells (100 μL $well^{-1}$) and incubated for 1 h. After washing with distilled water, 100 μL of peroxidase-labelled antimouse IgG (Nordic, Tilbur, the Netherlands) were added per well and incubation continued for 30 min. After washing, 50 μL $well^{-1}$ of 1 mg mL^{-1} diaminobenzidine (DAB) (Sigma Chemical Co., St Louis, MO, USA) in the appropriate buffer were added until brown foci were detected using an inverted microscope.

In order to demonstrate pH-dependent VHSV-induced cell fusion, the present authors used the syncitia formation procedure previously described

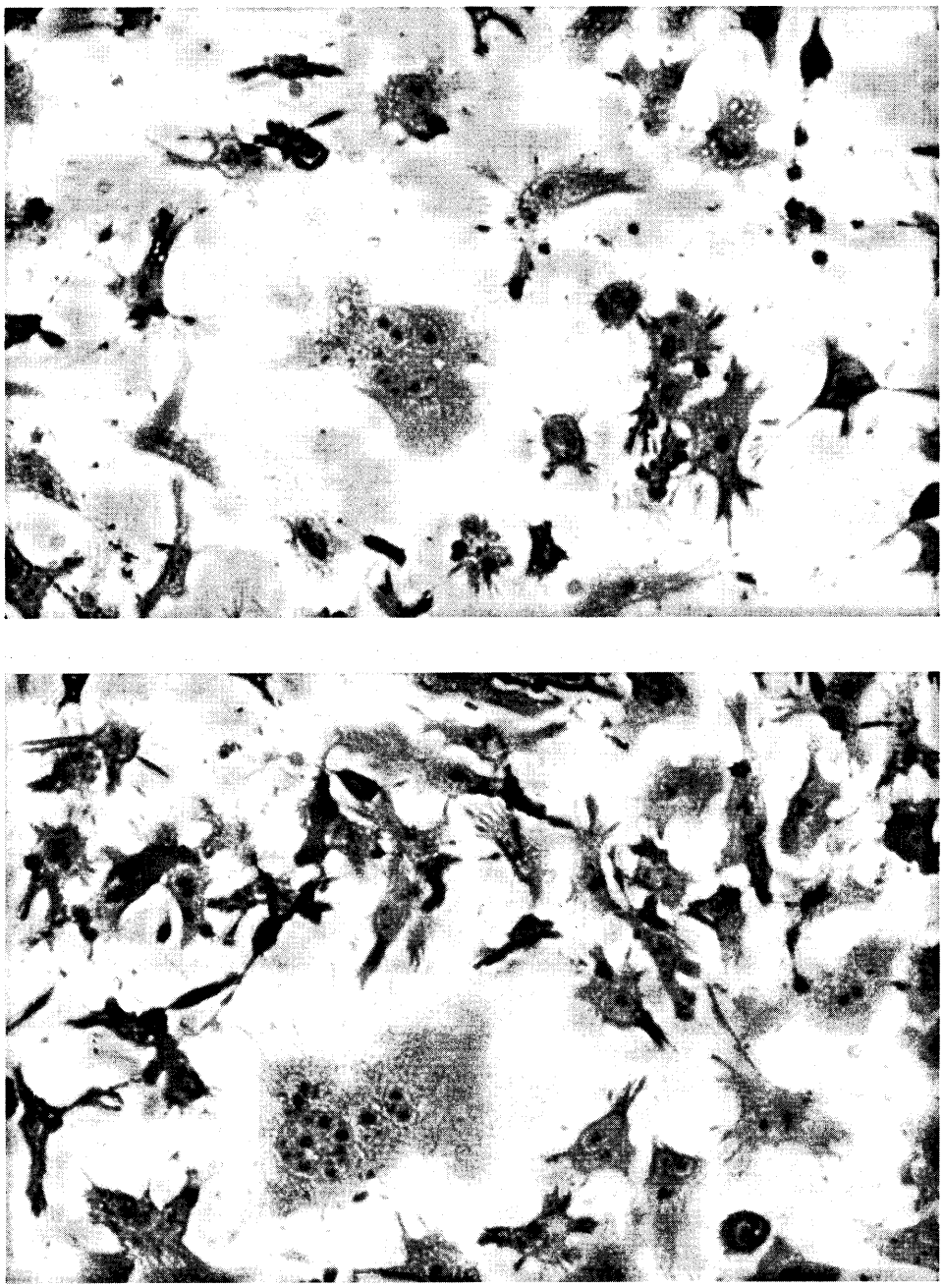

Figure 1 EPC-19 cells after exposure to pH 5.6. The EPC-19 cells were grown at 22 °C and the cell culture medium was replaced by medium at pH 5.6 for 30 min. The cells were fixed, stained and photographed as indicated: (a) after first passage; and (b) after five passages.

by Estepa & Coll (1997). Briefly, the EPC plates were washed with RPMI-1640 medium at pH 5.6 (obtained by using media without bicarbonate and buffered with 20 mM HEPES and 20 mM MES). After 30 min incubation at 14 °C with manual agitation, the cultures were fixed for 10 min with cold methanol, stained with Giemsa, washed and dried. The monolayers were photographed using a Nikon inverted microscope with a SBIG ST-7 camera (St Barbara Instrument Group, St Barbara, CA, USA) and the CCDOPS program VSSS1.09 (Microsoft Corp., Redmond, WA, USA) to analyse the resulting images.

Survivor colonies appeared in EPC cell monolayers about 2 months after VHSV infection (multiplicity of infection of 10^{-5} $TCID_{50}$) in a cell

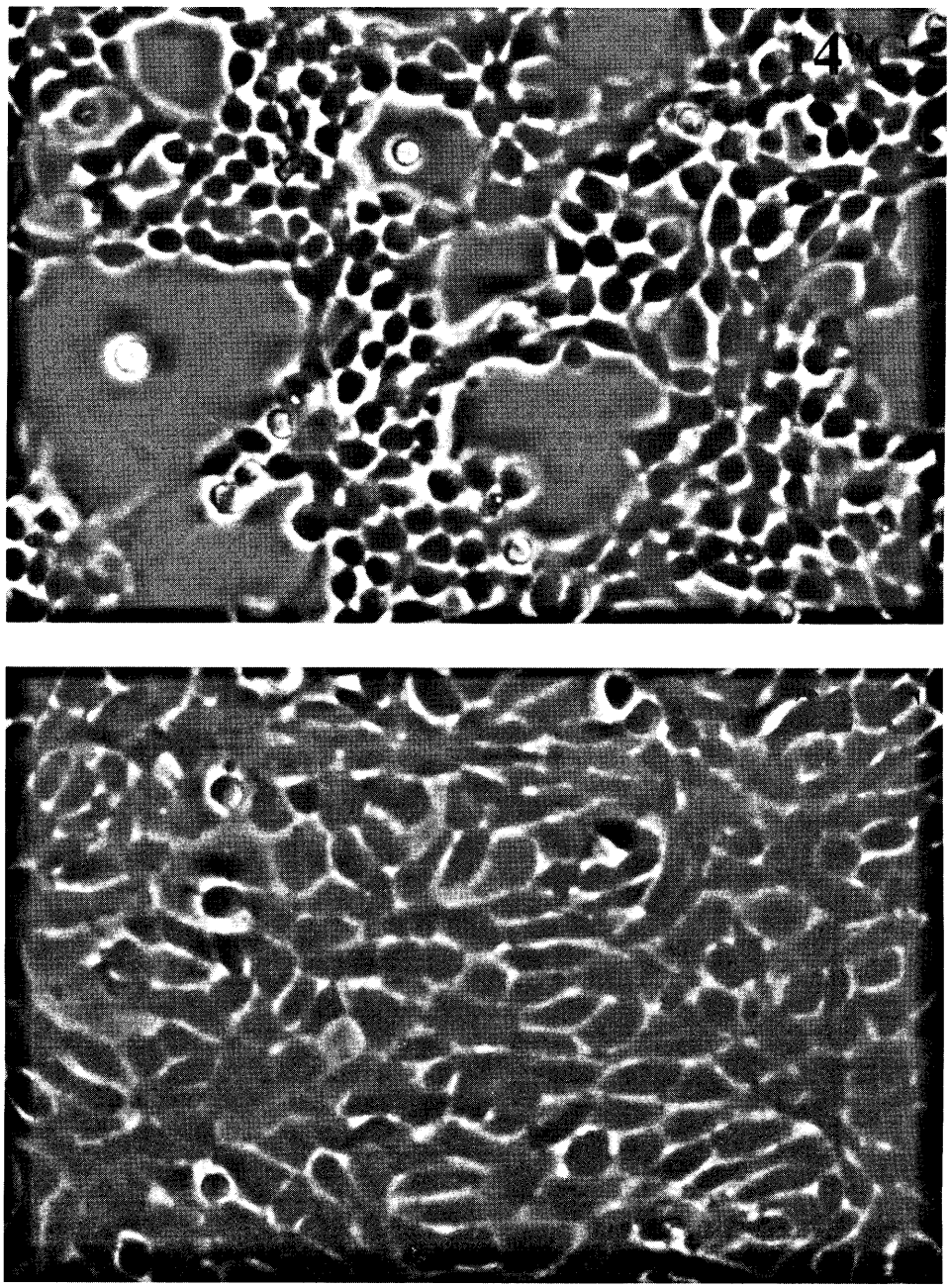

Figure 2 EPC-19 cells grown at (a) 14 °C and (b) at 22 °C.

Table 1 Staining of EPC-19 with a panel of anti-VHSV antibodies*

Antibody	Staining	Properties	Reference
IP1H3	+	Anti-G linear (aa399–4513)	Fernandez-Alonso *et al.* (1998)
3F1 A12	–	Neutralizing/conformational	N. Lorenzen, unpublished data
I10	+	Anti-G lineal (aa139–153)	Fernandez-Alonso *et al.* (1998).
C10	–	Neutralizing/conformational	Commercial
2C9	+	Anti-N	Sanz & Coll (1992)
αp2	–	Anti-G peptides p2(aa82–109)	Estepa & Coll (1996)
αp3	+	Anti-G peptides p3(aa110–121)	Estepa & Coll (1996)
αp4	+	Anti-G peptides p4(aa122–151)	Estepa & Coll (1996)
PAb	+ + +	Anti-VHSV	Present study
1G7	–	Anti-IgM trout	Sanchez, Coll & Dominguez (1991)

*After five passages in cell culture, EPC-19 cells were distributed in a 96-well plate and grown for a further 3–5 days at 14 °C. After fixing, the cells were stained with the different antibodies and DAB as indicated: (+) ≤ 20–50% of the cells stained; (+++) 100% of the cells stained; and (–) not stained. The synthetic peptides p2, p3 and p4 were obtained from Clontech (Palo Alto, CA, USA). The polyclonal antibodies antipeptides (αp) and anti-VHSV were obtained following procedures previously described in Estepa & Coll (1996). The neutralizing MAb 3F1 A12 was a gift from Dr N. Lorenzen. The neutralizing MAb C10 was obtained from Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France.

culture bottle of 150 cm^2. The cytopathic effect was allowed to proceed for 2 weeks at 14 °C, and then the cell culture medium was changed and the cells were grown at 20 °C until colonies were visible. The clones were picked with a Pasteur pipette and grown in replicates in two, 24-well plates, one clone per well for 5 days at 20 °C.

One of the replicated plates was used for the cell fusion assays. Figure 1 shows some of the syncitia formed in one of the fusion-positive clones. Only four colonies out of the 48 assayed showed positive fusion after lowering the pH. Two clones displayed 19.6% of the cells fused, whereas in two other clones, less than 2% of the cells fused. One of the clones (EPC-19) showing the maximal percentage of fusion was selected and cultured at 14 and 22 °C. Figure 2 shows the different appearance of the EPC-19 cell line at the two different temperatures. At 14 °C, the EPC cells grew poorly, but VHSV production was higher as shown by the titres of the virus released (data not shown), whereas the EPC cells grew much better at 22 °C.

That the EPC-19 cells expressed VHSV antigens after five passages was demonstrated by staining with a panel of monoclonal antibodies (MAbs) (Table 1). The percentage of cells expressing each epitope varied depending on the MAb used to stain the cells (Fig. 3). Not all the cells were stained, which might be because there were no true clones, there was a partial exposure of different epitopes or because of the different antigen expression in different cells. Thus, because the cells were fixed before staining, neutralizing MAbs 3F1 A12 and C10 did not react against their epitopes on protein G. However, the presence of protein G was demonstrated in 27–54% of the EPC-cells by staining with other MAbs (Fig. 3) which recognize the lineal epitopes I10 and IP1H3, respectively (Fernandez-Alonso *et al.* 1998). Thus, the percentage of cells which stained with these MAbs were similar to the percentage of fused cells (Fig. 3).

Polyclonal antibodies raised in mice against synthetic peptides belonging to protein G, αp2, αp3 and αp4 gave negative, positive and positive staining, respectively. This result confirms that the p2 peptide at pH 7 is inside the G protein and is only reactive with antibodies raised against it when the protein G is at pH 5.6, the optimal pH for fusion (Estepa & Coll 1997). In contrast, the p3 and p4 peptides are probably located outside the G protein at pH 7, and these do react with antibodies raised against them.

The presence of protein N of VHSV was also demonstrated by the positive staining obtained with MAb 2C9 (Sanz & Coll 1992). Because of the presence of the G protein in the EPC-19, these or analogous persistently infected cells can be used to generate large numbers of cells, and thus, to study cell fusion induced by the expression of the G protein in VHSV-infected cells.

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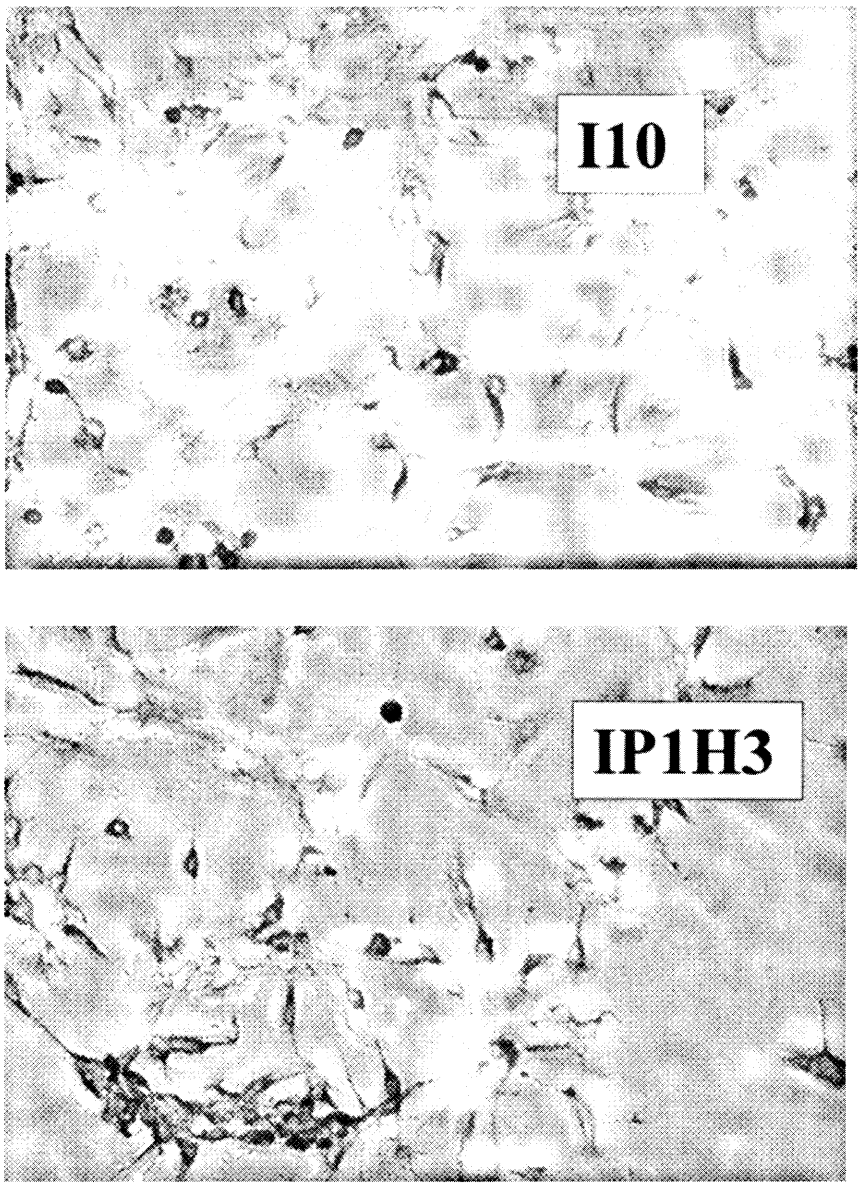

Figure 3 Staining of EPC-19 with MAb I10 and IP1H3.

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