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Enhanced detection of viral hemorrhagic septicemia virus (a salmonid rhabdovirus) by pretreatment of the virus with a combinatorial library-selected peptide

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Abstract

A 17-mer peptide (SAAEASAKATAEATAKG, p5) was selected by screening a combinatorial library for its ability to enhance in vitro the infectivity of viral hemorrhagic septicemia virus (VHSV), a salmonid rhabdovirus. Preincubation of VHSV samples with p5 at micromolar concentrations led to up to 5-fold increase of viral titers compared to untreated samples, as measured by a 1-day post-infection immunochemical focus assay. Treatment with p5 also increased VHSV titers when using the more traditional plaque and end-point dilution assays. Preincubation of p5 with infectious hematopoietic necrosis virus (another rhabdovirus of salmonids), but not with infectious pancreatic necrosis virus (birnavirus) also led to a similar increase in sensitivity. These results indicate that the addition of p5 may be used to improve the sensitivity of diagnostic tests for salmonid rhabdoviruses.

Keywords: Virus titration; Immunochemical assay; Enhancer peptide; Viral hemorrhagic septicemia virus; Infectious haematopoietic necrosis virus

1. Introduction

Viral hemorrhagic septicemia is a serious disease of rainbow trout in central and western Europe. The causative agent, the viral hemorrhagic septicemia virus (VHSV), belongs to the family *Rhabdoviridae*. Fish rhabdoviruses (VHSV and infectious haematopoietic necrosis virus, IHNV) pose a significant threat on European and American salmonid aquaculture as well as other farmed species elsewhere (Isshiki et al., 2001). Thus, there is a great need for rapid and sensitive diagnostic methods. Standard protocols for VHSV detection are carried out on monolayer cell cultures where samples from fish tissues, organs or fluids are tested for the induction of cytopathic effects (CPEs) (plaque formation or cell lysis which can be easily read by eye) on permissive cell lines (Amos, 1985; Amos et al., 1989; Batts and Winton, 1989). The major drawback of these approaches is

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that it takes from 5 to 9 days to obtain reliable results. In addition diagnosis usually requires further confirmation by subcultivation and virus-specific immunoassays (Amos, 1985; King et al., 2001).

In recent years a series of immunochemical as well as immunofluorescent staining methods for VHSV detection and quantitation have been developed (Falk et al., 1998; Hügin and Tang, 1999; Mourton et al., 1992; Zhou et al., 2001). Antigen capture ELISAs using monoclonal antibodies (MAbs) against the major nucleocapsid protein N were shown to be suitable for the detection of the virus in blood and in organ extracts (Mourton et al., 1992; Sanz and Coll, 1992). More recently, an immunochemical focus assay combining cell culture with the use of an anti-N MAb was established (Lorenzo et al., 1996). This assay takes only 2 days and it is reproducible and more sensitive than the ELISA method. This report describes how the sensitivity of this immunochemical focus assay and of other methods based on virus growth in cell culture can be improved by using a 17-mer peptide selected from a combinatorial peptide library for its capacity to enhance VHSV infectivity in cell culture (Mas et al., 2002, in press).

2. Materials and methods

2.1. Cell lines

Epithelioma papulosum cyprini (EPC) and rainbow trout gonad (RTG-2) cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). Both cells were grown in RPMI-1640 medium Dutch modification (GIBCO-BRL, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 1 mM sodium piruvate, 50 μg/ml gentamicin, 2 μg/ml anphotericin B (from ICN Biomedicals, Aurora, OH) and 10% fetal calf serum (FCS, Sigma, St. Louis, MO). The RTG-2 cell line was maintained at 20 °C and the EPC cell line at 25 °C.

2.2. Virus strains

VHSV strain 07.71 isolated originally in France (Le Berre et al., 1977) was used. VHSV was propagated in EPC cells in 2% FCS medium at 14 °C until CPE was complete. Cell debris were sedimented by centrifugation at $2500 \times g$ during 25 min at 4 °C and the virus-containing supernatant (extracellular virus) was collected. Other viruses employed in this study were the IHNV type strain (American Type Cell Culture Collection, VR-714) which was grown on EPC cells, and infectious pancreatic necrosis virus (IPNV) Sp strain (ATCC, VR-1318) which was grown on RTG-2 cells.

2.3. Preparation of intracellular VHSV

Confluent monolayers of RTG-2 cells were infected at a multiplicity of infection (moi) of 0.01 plaque forming units (pfu)/cell with the VHSV 07.71 strain. At 17-h post-infection, the culture medium was removed and the cell monolayer harvested in 5 ml of sterile distilled water and submitted to a series of freezing and thawing cycles to lyse the cells. Finally, cell debris were pelleted and aliquots of the supernatant stored at −70 °C until use.

2.4. Plaque assay for VHSV

EPC cells were grown on 24-well plates until 100% confluent and inoculated with 200 μl of serial dilutions of virus samples. After 1 h adsorption at 14 °C, the medium was replaced by 0.5 ml of 0.75% methyl cellulose (Sigma) in 2% FCS RPMI. Plates were incubated at 14 °C until full plaque formation (day 8 post-infection or later). After fixing and staining with a 0.1% crystal violet solution in formalin, the number of plaques were counted by eye. The results were expressed as pfu.

2.5. End point dilution assay for VHSV

Serial dilutions of VHSV samples in RPMI medium supplemented with 2% FCS were added to EPC cell monolayers in a microwell plate and incubated at 14 °C for 9 days. The presence of

virus was determined by eye reading of the CPE and confirmed by crystal violet staining of the wells. Determination of virus titer (expressed as TCID50) was done by the Reed-Münch end-point calculation method (Hierholzer and Killington, 1996; Svensson et al., 1999).

2.6. Immunochemical focus assay for VHSV, IHNV and IPNV

The immunochemical focus assay described earlier (Lorenzo et al., 1996) was used. Briefly, VHSV-infected cell lysates or culture supernatants were diluted in RPMI Dutch modification-2% FCS medium to an estimated 10^3 pfu/ml virus concentration and 100 µl were used to infect EPC cell monolayers on 96-well plates. At 26 h post infection cells were fixed by adding cold methanol. Clusters of infected cells (foci) were revealed by an anti-nucleocapsid protein MAb (2C9) and a peroxidase-conjugated rabbit anti-mouse secondary antibody (Sigma). For IHNV, a cocktail of mice ascites from each of 5 anti-nucleocapsid MAbs (1NH17W, 2NCO42C, 2NH105B, 1NCO27G, 14D) was used (Ristow and Arnzen, 1991). IPNV-infected cells were detected with an anti-IPNV MAb (2F12) (Dominguez et al., 1990). The color reaction reagent was diaminobenzidine (DAB, Sigma). Finally, the number of dark brown foci in each well was counted under the microscope. The results were expressed as foci forming units (ffu).

3. Results

3.1. Enhancement of VHSV infectivity by incubation of the virus with p5

VHSV was preincubated with increasing concentrations of p5 (0.2–5 µg/ml, equal to 0.13–3.2 µM) prior to inoculation of EPC or RTG-2 cells. In both cases the number of VHSV ffu increased 3–4-fold over the control samples when samples were treated with p5 (Fig. 1A). Although the enhancing effect of p5 was similar on the carp and the trout cell lines, the number of ffu was significantly higher ($\sim$ 3-fold) on EPC. Thus, EPC was usually chosen for further focus assay experiments.

The variation of the number of ffu with respect to the dilution of the inoculum and therefore to the moi showed that in the range of moi assayed there was a correlation between the amount of VHSV in the inoculum and the number of ffu obtained (not shown), supporting the validity of this method for VHSV titration.

In clinical specimens a significant amount of infectious units are intracellular virus particles. Thus, the effect of p5 incubation on extracellular VHSV (infected cell culture supernatant) and on intracellular VHSV (infected cells lysate) was analyzed (Fig. 1B). At 5 µg/ml of p5 the infectivity of both intra and extracellular VHSV was higher than the respective non-treated controls (100%). A larger increase was obtained with the p5-treated extracellular virus ($551 \pm 51\%$) than for the p5-treated intracellular virus ($212 \pm 72\%$).

3.2. Improved reliability of VHSV detection with p5

To confirm the increase of infectivity of intracellular VHSV with p5, samples from infected RTG-2 cells diluted 1000-fold to be among the low range of ffu values (1–10 ffu) were analyzed, with or without previous treatment with p5 (Table 1). Replicates of each type of sample were assayed and those wells with $\geqslant 1$ brown foci were scored as a positive result. In untreated samples, 6% of the results turned out to be negative. On the contrary, in the p5-treated series a 100% positive result was achieved with an average 2.2-fold increase in ffu. In a similar experiment, dilutions of the infected cell lysate slightly under the detection limit of the focus assay were treated either for a short (1 h) or for a long (16 h) period of time with p5 and tested for the formation of foci (Table 1, 1:20 000 sample dilution). All of the control (non-treated) viral samples were negative. In contrast, 70% of the samples in the short (1 h) treatment with p5 and 100% of the samples in the long (16 h) treatment with p5 were positive. The average number of foci in positive wells was higher (about 2-fold) in the longer p5 incubation series.

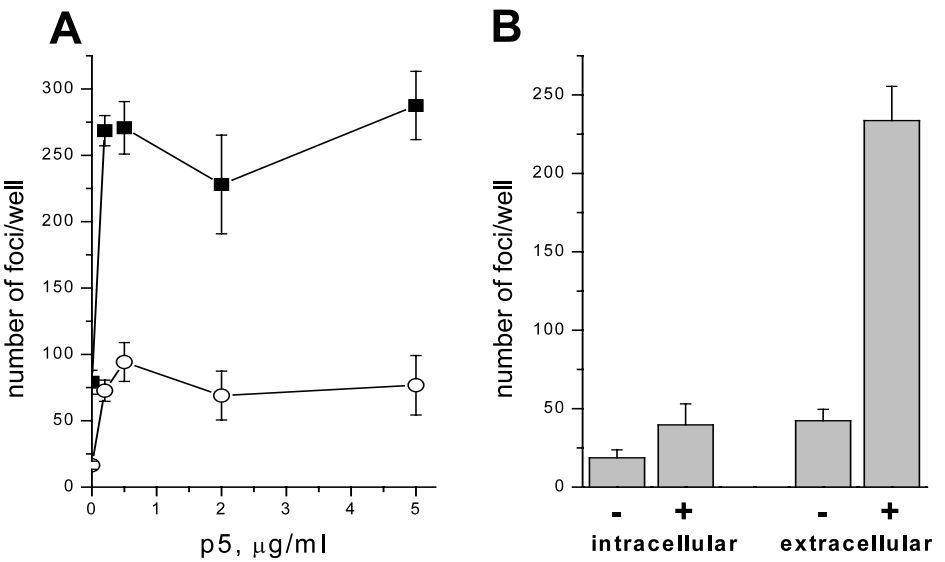

Fig. 1. (A) Detection of VHSV foci after treatment with p5 on two fish cell lines. Virus samples were preincubated with the indicated concentrations of p5 for 7 h at 4 °C. The focus immunochemical assay was performed on a 96-well plate as described in methods. Results are expressed as the average and S.D. from 4 wells. The number of foci/well without p5 were 79±9 for EPC (■) and 16±3 for RTG-2 (○). (B) Effect of p5 at 5 μg/ml (+) on intracellular and extracellular VHSV. Number of foci in control (−) samples: 19±5 for intracellular VHSV; 42±7 for extracellular VHSV.

3.3. Effect of p5 as determined by other methods of virus titration

The immunochemical focus assay was compared to the plaque assay and the end-point dilution methods in parallel experiments. Virus from an infected cell lysate (intracellular VHSV) was used. Infection was stopped at 26 h p.i. for focus detection, while infected cells were maintained under methyl cellulose for 8 days to allow plaque formation. In the end-point dilution assays the incubation time was 9 days. The effect of p5 on VHSV in a plaque assay is illustrated in Fig. 2. An average 80% and a 89% more pfu and TCID50, respectively, were obtained in the presence of p5 compared to the titers in the absence of p5 (Table 2). The average enhancing factor of p5 in the focus assay was slightly lower (59%). However, the end-point dilution assay (9 days) as well as the plaque assay (8 days) are much more time consuming than

Table 1
Improved detection of intracellular VHSV by treatment of the samples with p5

	Sample dilution			
	1:1000 ($n = 18$)		1:20 000 ($n = 10$)	
	Positive wells	Foci/well[a]	Positive wells (%)	Foci/well[a]
VHSV control	94%	2.1	0	–
VHSV+p5 (1 h)	n.d.	n.d.	70	1.3
VHSV+p5 (16 h)	100%	4.7	100	2.5

EPC cells grown in microplate wells were infected with 100 μl of a 1×10^{-3} (1:1000 dilution) or 5×10^{-5} (1:20 000 dilution) of a VHSV-infected RTG-2 lysate (intracellular VHSV). Virus samples were treated either for 1 h or for 16 h with 10 μg/ml p5. n, Number of samples analyzed. The percentage of VHSV-positive results (one or more foci in the well) is shown. n.d., Not determined.

[a] Mean number of foci in positive wells.

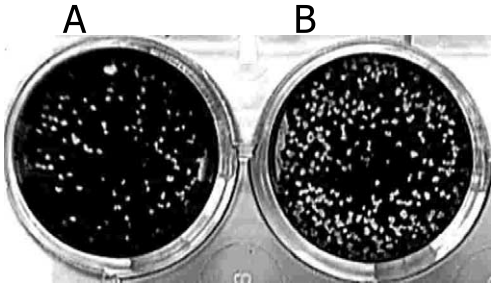

Fig. 2. Increased VHSV infectivity by treatment of intracellular virus samples with p5 in a plaque forming assay. VHSV from infected RTG-2 cells lysate was incubated with or without 10 μg/ml of p5 for 16 h at 4 °C. Plaque assay was performed on EPC cells, as described in methods. Cell monolayers were fixed and stained at day 8 post-infection. (A) control VHSV, 48 plaques; (B) p5-treated VHSV, 85 plaques.

the focus assay (2 days), and the overall virus titers in pfu/ml or TCID50/ml were lower (less than a half than titers in ffu/ml).

3.4. Effect of p5 treatment on other rainbow trout viruses

When tested for foci formation on another salmonid rhabdovirus, IHNV, p5 also showed a ~5-fold enhancer effect as described for VHSV (Fig. 3) whereas on IPNV (a fish birnavirus) infectivity was not significantly modified by p5 (135±6%). The experiments shown in Fig. 3 were carried out using supernatants from infected cell cultures (extracellular virus) and therefore ~500% with respect to non-treated virus were obtained for virus+p5 as seen in Fig. 1B.

4. Discussion

The sensitivity of an immunochemical focus assay for VHSV described previously (Lorenzo et al., 1996) was increased by preincubation of the virus with a 17-mer peptide (p5) selected from a combinatorial library for its VHSV infectivity enhancing capacity (Mas et al., submitted for publication). The immunochemical focus assay has a much shorted detection period than the plaque or TCID50 assays (2 days vs 8–9 days) and by using the enhancer peptide it becomes ~60% more sensitive for intracellular virus and 3–5-fold more sensitive for extracellular virus detection. This difference may be due to the presence of non-fully mature virions inside the infected cells which might be less or not at all susceptible to the effect of p5. In our hands, the p5-improved focus assay is the most reliable method for VHSV detection. In fact, our studies showed that in samples containing small numbers of virus particles VHSV remained undetected unless the samples were treated with p5. Furthermore, the p5-induced enhanced detection of VHSV was observed irrespective of the method employed (focus, plaque, or end-point dilution assays).

The mechanism of action of p5 is currently being investigated in our laboratory. Preliminary experimental evidence supports a model where a direct interaction between the p5 and the virus envelope glycoprotein (protein G) occurs (Mas et al., submitted for publication). Shorter peptides than p5 have also been reported to increase the infectivity of bovine leukemia virus (Voneche et

Table 2
VHSV titers and enhancing effect of p5 as determined on EPC cells by focus, plaque and end-point assays

Method	Virus titers × 10^{-5}			
	−p5 (100%)	+p5		
			% of the control	Time, days
Focus assay, ffu/ml	0.78	1.21	159	2
Plaque assay, pfu/ml	0.22	0.41	180	8
End point, TCID50/ml	0.27	0.51	189	9

Peptide p5 at 10 μg/ml was preincubated (16 h, 4 °C) with ~10^3 ffu/ml of VHSV from an infected RTG-2 cells lysate. The assays were carried out on EPC cell monolayers. Results were expressed as percentage of the control as calculated by the formula (ffu in the presence of p5/ffu in the absence of p5) × 100.

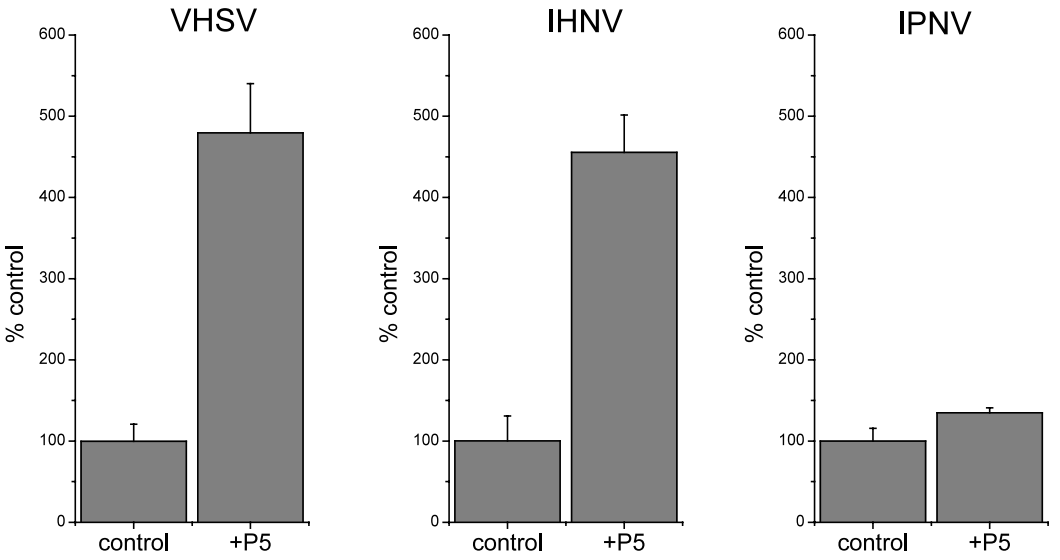

Fig. 3. Comparison of the effect of p5 on VHSV, IHNV and IPNV infectivity. VHSV and IPNV from infected cells supernatant were inoculated into RTG-2 cell monolayers. IHNV samples also from an infected cell culture supernatant were tested on EPC cells. +p5, incubation with 10 μg/ml p5 for 10 h prior to infection. VHSV-infected cells were analyzed at 26 h p.i. IHNV-infected cells were incubated for 37 h before fixing. IPNV-infected cells were incubated for 33 h before fixing. Average numbers of foci and standard deviations in untreated samples (100%) were: VHSV, 14±3; IHNV, 12±4; IPNV, 23±4. In p5-treated samples: VHSV+p5, 66±8; IHNV+p5, 56±6; IPNV+p5, 31±1.

al., 1993) due to their membrane fusion enhancement capacity.

Immunochemical focus assays based on microscopical detection of stained clusters of infected cells (so called foci) are rapid and reliable methods for virus quantitation when compared to the standard plaque assay (Hügin and Tang, 1999; Zhou et al., 2001). Moreover, approaches based on specific antibody reaction have been proven useful for the detection of other salmonid viruses which do not induce a distinct CPE in cell culture as it is the case of infectious salmon anaemia virus (Falk et al., 1998). These methods can be improved either by using more sensitive reagents as in a chemiluminescent assay reported recently (Richards and Watson, 2001) or by the treatment of the samples with compounds which could cause an increase in the number of ffu as it is described in here.

In conclusion, the sensitivity an immunochemical multiwell plate focus assay for VHSV was improved significantly by the addition of micromolar concentrations of p5, a 17-mer synthetic peptide. In comparing the focus assay and other more conventional virus detection methods, the p5-improved immunochemical focus assay has been shown to be the most convenient regarding sensitivity, reliability, time consumption and simplicity, providing fast and easy measurements of VHSV titers. The fact that p5 increases detection of VHSV and IHNV, both of them belonging to the *Rhabdoviridae* family and does not increase detection of IPNV (a salmonid birnavirus), suggests that p5 can be used to enhance fish rhabdovirus detection.

Should these results be confirmed on clinical specimens, p5 may become a useful tool to improve diagnosis of the most important rhabdoviral diseases in salmonid aquaculture.

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