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Veterinary Immunology and Immunopathology 99 (2004) 143–152
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Characterisation of the syncytia formed by VHS salmonid rhabdovirus G gene transfected cells

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Received 6 May 2003; received in revised form 26 January 2004; accepted 26 January 2004

Abstract

Protein G expression and cell-to-cell fusion of cells transfected with the G gene of viral haemorrhagic septicaemia virus (VHSV) has been characterised. The presence of protein G in the membrane of transfected cells was confirmed by staining with Abs (Abs) and FACS. The subsequent formation of syncytia by membrane fusion of transfected cells required transfection with a wild type G gene and a low pH step. Mice Abs made against the protein G regions involved in fusion and neutralising monoclonal Abs (MAbs) as well as MAbs against some linear epitopes inhibited syncytia formation, thus confirming that syncytia formation was G-dependent. Similarly, Abs from trout immunised with purified VHSV or protein G inhibited syncytia formation whereas Abs from non-immunised or non-infected animals did not. Abs from mice or trout with the highest neutralisation titres also showed the highest percentage of inhibition of syncytia. While the main utility of these observations might be to further the understanding of the complex trout antibody response against VHSV and in the follow up of VHSV immunisation attempts, they may also have some future diagnostic potential for countries were work with VHSV is not allowed.

Keywords: VHSV (viral haemorrhagic septicaemia virus); Syncytia; Fusion; Protein G; Mutants

1. Introduction

Despite the advances obtained in recent years, the trout antibody response to viral haemorrhagic septicaemia virus (VHSV) infection is still a matter of study (Lorenzen et al., 1993, 1999). To certify VHSV-free trout stocks or to follow up immunisation to VHSV during vaccination attempts, the methods for estimating protein G-specific trout Abs to VHSV are based on in vitro neutralisation (Jorgensen et al., 1991), microneutralization (Lorenzo et al., 1996) or in binding to VHSV-captured ELISAs (Olesen et al., 1991). Binding to solid-phase recombinant fragments of the protein G (Rocha et al., 2002) might also be used. The application of other methods like those employed to study VHSV-infected cell-to-cell fusion (Estepa and Coll, 1997; Fernandez-Alonso and Coll, 1999), could help to study the antibody response of trout to VHSV and to develop new methods to assay for trout anti-VHSV Abs.

The protein G of VHSV binds to the host cell receptor(s) and then VHSV is endocytosed and fuses with the host membranes (Bearzotti et al., 1999;

Abbreviations: VHSV, viral haemorrhagic septicaemia virus; EPC, epithelioma papulosum cyprini; CMV, cytomegalovirus; βgal, β galactosidase; PEG, polyethyleneglycol; FDG, dodecanoyl aminofluorescein

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0165-2427/$ – see front matter
doi:10.1016/j.vetimm.2004.01.006

Coll, 1995; Gaudin et al., 1993) at the low pH of the endosomes (Coll, 1999; Gaudin et al., 1999a,b). As a consequence of the expression of the G protein in the host cellular membranes and its fusion properties, VHSV-infected (Estepa and Coll, 1997; Fernandez-Alonso and Coll, 1999) or G gene transfected (Lecocq-Xhonneux et al., 1994) cells, form syncytia when exposed to a pH slightly lower than neutral. Since the natural pathway of viral-host membranes fusion can be mimicked by cell-to-cell fusion of cells transfected with the G gene after lowering the pH of the cell culture medium (Estepa and Coll, 1997; Estepa et al., 2001; Lecocq-Xhonneux et al., 1994), we have undertaken the characterisation of the syncytia formed by G gene transfected cells.

2. Materials and methods

2.1. VHSV and cell culture

The virus used was the isolate VHSV-07.71 (LeBerre et al., 1977). Epithelioma papulosum cyprini (EPC) cell culture techniques and virus polyethylene glycol (PEG) concentration were performed as described by DeKinkelin (1972) and modified by Basurco and Coll (1989). To be used for inoculum in the neutralisation assays, supernatants from VHSV 07.71 infected EPC were clarified by centrifugation at 20,000 × *g* during 20 min, and the pellets kept frozen at −70 °C until used.

2.2. Plasmids

The plasmids used were the pCMVβ (Clontech Labs, Palo Alto, CA) containing the β galactosidase (βgal) gene of *E. coli* under the control of the cytomegalovirus early promoter (CMV), the G3-pcDNAI/Amp promoter (Fernandez-Alonso et al., 1999a) and the pMCV1.4-G (Rocha et al., in press), both of them containing the protein G gene from the viral haemorrhagic septicaemia virus under the short and the long CMV promoter, respectively. The plasmids were used to transform *E. coli* XL2 and Top10, respectively. Large amounts of plasmid were prepared from *E. coli* pellets using the Wizard plus Megaprep DNA purification system (Promega, Madison, USA). Plasmid solutions were adjusted to 0.5–1 mg/ml of total DNA (absorbance at 260 nm).

2.3. Site-directed mutagenesis

The in vitro site-directed mutagenesis method based on the Quick-Change technique (Stratagene, La Jolla, CA, USA) to make point mutations was followed. Briefly, a supercoiled double-stranded pMCV1.4-G plasmid and two oligonucleotide primers of 15 nucleotides each with the mutation 5′ 'C256G' (P86A) and with the 5′ 'G293C' (G98A) were used. The oligonucleotide primers were extended during thermal cycling with Pfu turbo DNA polymerase (Stratagene) generating a mutant plasmid in a nicked circle form. Then DpnI endonuclease treatment specific for methylated DNA was carried out to digest the parental DNA. The nicked plasmid DNA incorporating the desired mutation was then transformed into XL1-blue competent cells (Stratagene). Plasmids were produced in large amounts and purified as described before. Confirmation of the correct mutated construct was achieved by sequencing plasmid DNA across the changed region (Cytomix, Cambridge, England).

2.4. Production of anti-protein G and anti-frg11 (amino acid 56–110 of protein G) polyclonal Abs in mice

Each female BALB/c mouse were first injected with 20 μg of purified protein G (Perez et al., 1998), G derived synthetic peptides p2 (amino acid 82–109) or p3 (amino acid 110–122) or purified recombinant frg11 (amino acid 56–110) of the protein G which is related to VHSV fusion (Estepa et al., 2001; Rocha et al., 2002) in Freund's complete adjuvant. VHSV was also injected in saponin. Concentrated VHSV (30 μg) was sonicated in the presence of 30 μg of saponin (Superfos, Quil A), 600 μg of cholesterol and 6 μg of phosphatidylcholine (Larsson et al., 1993; Morein et al., 1984) in 1 ml of distilled water. To each mouse 200 μl of the mixture were injected intraperitoneally. Then 4-monthly injections with the same antigens in Freund's incomplete adjuvant or saponin were carried out. To obtain ~40 ml of pooled diluted ascites, each of two groups of three immunised mice per antigen were intraperitoneally injected with $(0.5-2) \times 10^6$ viable myeloma X63/Ag8653 cells per mouse. Mice ascites was then

obtained by injection of physiological saline a few days later and pooled. The pooled ascites was passed throughout a 3 cm × 10 cm Sepharose column (Pharmacia) with bound *E. coli* protein extract (~10 mg/ml) and rabbit serum (~10 mg/ml) to adsorb background Abs. The ascites was then 10-fold concentrated with 40% ammonium sulphate and dialysed against 10 mM sodium phosphate, 150 mM NaCl, pH 7.2 (PBS). Purity as tested by PAGE gave two single Coomassie blue stained bands at 50 and 24 kDa, respectively.

2.5. Production of anti-VHSV polyclonal Abs in trout

Trout (200–500 g body weight) were held in 100 l aquaria at 12–18 °C. Trout to be immunised were distributed in two groups.

One group, trouts 1 and 2 were immunised with concentrated VHSV + purified G and Freund's. Trout were injected intraperitoneally four times during 3 months. To each trout, 30 μg of heat killed (37 °C, 30 min) PEG-concentrated VHSV + 30 μg of G diluted 1:1 with complete Freund's adjuvant were injected for the first injection and diluted 1:1 with incomplete Freund's for the rest of the injections. Neutralisation titres of trout sera 1 and 2 were ~100.

In the second group, trout 3 was immunised with concentrated VHSV and saponin. Concentrated VHSV (30 μg) was sonicated in the presence of 30 μg of saponin (Superfos, Quil A), 600 μg of cholesterol and 6 μg of phosphatidylcholine (Larsson et al., 1993; Morein et al., 1984) in 1 ml of distilled water. One millilitre of the mixture was injected intraperitoneally four times during 3 months. Neutralisation titres of trout sera 3 was ~2000.

Three other trout sera with neutralisation titres ~100,000 were kindly provided by Dr. Kinkelin (Jouy-in-Josas, France) and Dr. Lorenzen (Aarhus, Denmark).

As control trout serum, 15 sera from healthy non-infected non-immunised trout (500 g of body weight) were obtained from a farm with no previous history of VHSV infections (Escuela de Montes, Madrid, Spain).

2.6. VHSV microneutralization assay

Briefly, 10^3 $TCID_{50}$/ml of VHSV 07.71 were incubated overnight at 4 °C with serial dilutions of trout serum. Then cultures of EPC cells in 96-well plates were infected with 100 μl of the different virus/antibody mixtures, adsorbed during 1 h at 14 °C, washed and incubated overnight at 14 °C. To detect the N protein of VHSV in the cell monolayers, after fixing, monoclonal antibody (MAb) 2C9, peroxidase-labelled anti-mouse IgG (Nordic, Tilburg, The Netherlands) and diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MI) were used. The neutralisation titre was defined as the reciprocal of the maximal dilution that reduced to background values the number of DAB positive foci (Lorenzo et al., 1996).

2.7. Transfection of EPC cells

Epithelioma papulosum cyprini cells (Fijan et al., 1983) were grown in 24-well plates at 28 °C with 500 μl of RPMI Dutch modified culture medium buffered with 20 mM HEPES and supplemented with 10% of foetal calf serum. The cells were transfected the next day when about 70% confluent (>600,000 cells/well). The plasmids were complexed with fugene 6 (Roche, Barcelona, Spain) during 15 min in 100 μl of RPMI and then added to the wells containing EPC monolayers and 400 μl of cell culture medium.

2.8. Chemiluminescent assays

To assay for βgal with a chemiluminescent substrate, the "Gal screen assay" from Tropix (Bedford, MA, USA) was used according to the manufacturer instructions. Briefly, after removing the cell culture medium, 200 μl of lysis buffer were added to the transfected-incubated EPC cells and the wells were agitated. The bioluminescence was estimated 60 min later at 1 s (counts per second, cps) in a Minilumat LB9506 apparatus (EG & Berthold, Madrid, Spain).

2.9. β galactosidase Xgal assay

After transfection, EPC cell monolayers were fixed during 5 min with 1% glutaraldehyde in 20 mM phosphate buffered saline pH 7.4 (PBS) and stained with 2 mg/ml of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (Xgal) in 5 mM ferrocyanide II, 5 mM

ferrocyanide III, 2 mM $MgCl_2$ in PBS, at 37 °C until blue cells were visible.

2.10. Enzyme immunoassays in solid-phase

Wells from high binding plates (Costar) were coated with 100 μl of PEG-concentrated VHSV (to assay for anti-G Abs binding) in distilled water and allowed to dry overnight at 37 °C. All the samples and reagents were in dilution buffer (0.5% bovine serum albumin, 0.3% rabbit serum, 0.1% Tween-20, 0.01% merthiolate, 0.005% phenol red in phosphate buffered saline). To assay for anti-G Abs binding, anti-G MAbs of known target epitopes were added to the plates containing solid-phase VHSV at different pHs and incubated during 1 h. After washing, 1000-fold diluted horseradish peroxidase-conjugated rabbit-anti-mouse antibody (Sigma Chemical Co., St. Louis, MI) was added and the colour reaction developed by adding 1 mg/ml *o*-phenylenediamine in citrate buffer containing 3 mM H_2O_2. Absorbance (*A*) at 492 and 620 nm for estimation of each individual well background were measured in an ELISA reader (Anthos, LabTec Inst.).

2.11. Flow cytometry assays (FACS)

To assay for the expression of protein G in the surface of transfected EPC cells, G3-pcDNA/Amp and/or pMCV1.4-G transfected cells were incubated for 1 h with rabbit-anti-G Abs (gift of Dr. Niels Lorenzen, Denmark), in RPMI medium containing 2% rabbit serum, 2% goat serum and 2% *E. coli* extract. After washing, the cells were incubated during 30 min with fluorescent goat anti-mouse Fab'2 fragment (Caltag, S. Francisco, CA, USA) and detached from the wells with FACS buffer (Beckton-Dickinson). On the same day of harvest and staining, 5000 cells were analysed by flow cytometry in a Beckton-Dickinson (San Jose, CA, USA) FACScan apparatus using the LYSYS II vs 1.0 program for analysis. Fluorescence was captured in the FL1 region (514–545 nm, green).

For the β galactosidase FDG assay, pCMVβ transfected EPC cell monolayers were incubated during 2 min at 28 °C with 1 mM dodecanoyl aminofluorescein (FDG) in 1% DMSO, 1% ethanol in 100 μl. After washing at 4 °C, the cells were detached from the wells in 1 ml of FACS buffer (Beckton Dickinson) and incubated during 2 h at 4 °C before analysis with the FACScan apparatus.

2.12. Fusion and fusion inhibition assays

To assay for fusion, we followed the optimal conditions of pH, time and temperature for VHSV-infected EPC cell fusion described earlier (Estepa and Coll, 1997). To assay for inhibition of low pH-induced cell-to-cell fusion, EPC cells were plated in 24-well plates (about 500,000 cells/well), and the next day they were transfected with 0.6 μg of the plasmid G3-pcDNAI/Amp complexed with 2 μl of fugene in 100 μl following the methods described before (Fernandez-Alonso et al., 1999a; Lopez et al., 2001; Rocha et al., 2002). On parallel plates, Abs were serially diluted in RPMI-1640 cell culture medium (without bicarbonate) buffered with 20 mM of HEPES and 20 mM MES (Sigma Chemical Co., St. Louis, MI) at pH 6 and incubated overnight at 4 °C. Next day, the EPC plates were washed and the serial dilutions of Abs were pipetted into the wells containing the EPC cell transfected monolayers. After 30 min of incubation, the cultures were washed with cell culture medium and incubated during 2 h at 14 °C with RPMI-1640 (without bicarbonate) buffered with 20 mM of HEPES and 20 mM MES at pH 7.6. After the final washing step, the EPC cell monolayers were fixed in cold methanol during 10 min, washed with distilled water, dried and stained with Giemsa. The number of nuclei in syncytia and the number of nuclei in cells were counted in about 400 cells/well. Results were then calculated as percentage of nuclei in syncytia (nuclei in syncytia, %) by the formula, number of nuclei in syncytia/total number of nuclei × 100. Non-transfected EPC cell monolayers treated in parallel with transfected EPC cell monolayers showed 1.05 ± 0.4% ($n = 6$) of nuclei in syncytia. Results were also expressed as relative number of nuclei in syncytia either by the formula percentage of nuclei in syncytia at a given pH/percentage of syncytia at pH 5, to compare fusion at different pHs (Fig. 3) or percentage of nuclei in syncytia in the presence of a given dilution of Abs/percentage of nuclei in syncytia in the absence of Abs, to compare inhibition of fusion by Abs (Figs. 4 and 5). The titres of inhibition of fusion were defined as the inverse of the dilution at which the number of syncytia were reduced to 50% of the initial number.

3. Results

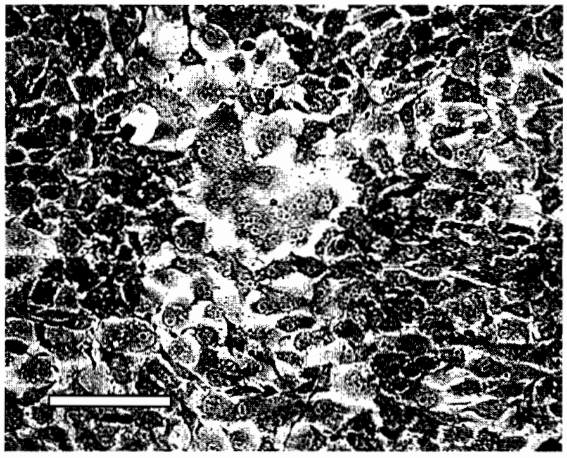
3.1. Optimisation of the fusion assay

Fig. 1A shows the morphological appearance of G gene transfected EPC monolayers after being exposed to pH 6, fixed and stained. Syncytia containing from 3 to 25 nuclei were found scattered among individual EPC cells. Most of the syncytia contained 3–4 nuclei when G3-pcDNAI/Amp was used for transfection whereas syncytia containing an average of 14 nuclei were found when pMCV1.4-G was used for transfection (Fig. 1B). The size and number of syncytia increased about 30–40% when the low pH exposed transfected cells were further incubated during two more hours at neutral pH (not shown). To avoid cell detachment during the low pH treatment step of the assay, the EPC cell monolayers have to be plated at least 24 h before transfection and 48 h before the low pH treatment. The syncytia were obtained at temperatures ~14–20 °C (buffers atemperated, plates at low temperature, etc.). Syncytia were not obtained at temperatures higher than 20 °C (not shown).

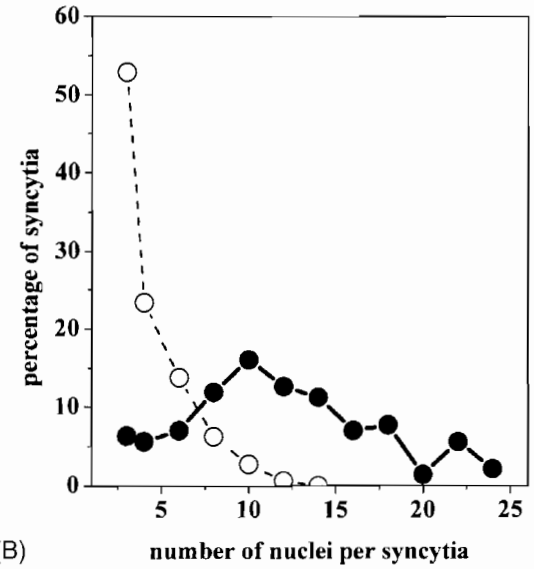
The highest β galactosidase (βgal) expression in EPC transfected cells with fugene-pCMVβ was obtained when using 0.6 μg of pCMVβ per well whether the βgal activity was estimated by the percentage of Xgal stained cells or by the βgal activity measured by luminescence or by the FACS-FDG assay (Fig. 2A). Similarly, the highest numbers of syncytia were also obtained when using 0.6 μg of G3-pcDNAI/Amp or pMCV1.4-G per well of a 24-well plate in 400 μl of medium with ~500,000 EPC cells plated the day before transfection. Higher or lower DNA concentrations showed decreasing numbers of syncytia to near background levels (Fig. 2B). Similar profiles were obtained if fusion was estimated by counting the number of syncytia per well or the percentage of nuclei present in syncytia (Fig. 2B).

3.2. Formation of syncytia was wild type G-dependent

To confirm the dependence of syncytia formation on the presence of protein G in the membrane of transfected EPC cells, we mutated the G gene in regions implicated in fusion. As suggested by previous work,



(A)



(B)

Fig. 1. Micrograph (A) and size distribution (B) of syncytia of G transfected EPC monolayers after incubation at pH 6. About 500,000 cells/well of a 24-well plate were plated and next day transfected with 0.6 μg of G3-pcDNA/Amp (○) or pMCV1.4-G (●) complexed with 2 μl of fugene in 400 μl of medium (B). Two days later the monolayers were incubated during 30 min at pH 6 and 20 °C, washed, incubated 2 h more at neutral pH, fixed and stained. White bar is 60 μm. A central syncytia is shown (A). The number of nuclei per syncytia (size of syncytia) were estimated in 200 syncytia per point and the percentage distribution of sizes calculated. Averages from two different experiments are shown in (B).

the region from the amino acid 82 to 109 of the protein G participates in phospholipid binding and G-dependent fusion (Estepa and Coll, 1996; Estepa et al., 2001;

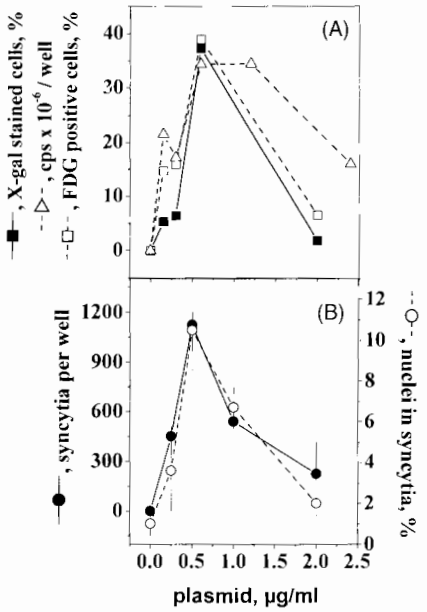


Fig. 2. Activity of βgal (A) and syncytia (B) detected in EPC cell monolayers after transfection with several concentrations of plasmids. (A) EPC cells were transfected with different concentrations of the plasmid pCMVβ after complexing with 2 μl of fugene. Activity of βgal was assayed by staining with Xgal, by bioluminescence or by staining with FDG (methods): (■) percentage of Xgal positive cells; (□) βgal activity per well as measured by bioluminescence; (△) number of FDG positive cells per well as estimated by FACS. (B) EPC cells were transfected with different concentrations of the plasmids G3-pcDNA/Amp or pMCV1.4-G after complexing with 2 μl of fugene. Number of syncytia per well and number of nuclei in syncytia opr cells in 400 nuclei per well were counted after fixing and staining the monolayers. The number of nuclei in syncytia were expressed in percentage of nuclei in syncytia (nuclei in syncytia, %) by the formula, number of nuclei in syncytia/total number of nuclei × 100. Similar results were obtained with G3-pcDNA/Amp or pMCV1.4-G: (●) number of syncytia per well: (○) percentage of nuclei in syncytia.

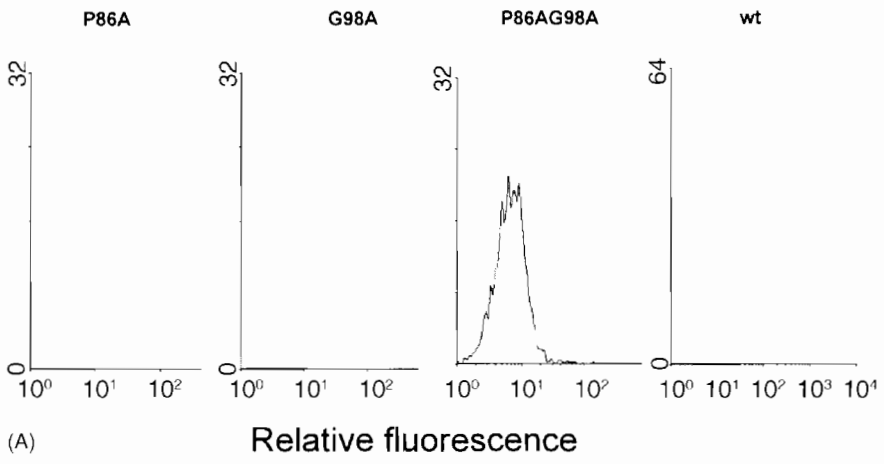
Nuñez et al., 1998). Because proline 86 and glycine 98 seemed to be some of the most salient features of this domain as determined by modelling (Estepa et al., 1999), we mutated both positions either by single or double mutations (P86A and G98A), as the most likely positions that could render mutants interfering with fusion. At the optimal transfection conditions, all the mutants obtained showed staining with anti-G Abs by FACS (Fig. 3A). Wild type G and the mutant P79A (outside the phospholipid binding region), showed a characteristic pH-dependent profile of fusion. The P86A and G98A mutants either single or double were fusion defective at all the pHs tested (from 5 to 7.3), thus confirming that syncytia formation required the presence of wild type protein G in the membrane of transfected cells (Fig. 3B).

3.3. Formation of syncytia in the presence of mice Abs

Since binding of anti-G Abs could vary at the low pH required to form the syncytia, we first analysed that possibility by ELISA using solid-phase VHSV. Abs to p2 only bound VHSV at pH 6 confirming data published before (Estepa and Coll, 1996), whereas Abs to frg11 showed a pH 7.5/6 ratio of binding of 2.2 ± 0.2 ($n = 4$). MAbs C10, 3F1A12, IP1H3 and I10 showed ratios of 1.3 ± 0.1, 1.4 ± 0.2, 1.4 ± 0.2 and 1.4 ± 0.2 ($n = 5$), respectively. The anti-N MAb 2C9 (against the N protein of VHSV) showed pH binding ratios of 0.96 ± 0.1, $n = 2$, similar to those obtained with mice polyclonal Abs. Therefore, except the Abs to p2 and frg11, all the other Abs used showed only small differences between binding to VHSV at pH 7.5 with respect to binding at pH 6.

The percentage of nuclei in syncytia formed by G gene transfected EPC cell monolayers when lowering the pH in the absence of Abs was $25.3 \pm 6.2\%$ ($n = 3$). Similar results were obtained when the assay was performed in the presence of mice ascites containing antibodies against an irrelevant antigen or in the presence of mice ascites containing anti-N MAb 2C9. The percentage of nuclei in syncytia formed at the above mentioned conditions were taken as the control value of syncytia formation (100%). Using the same assay, the percentages of nuclei in syncytia were then estimated in the presence of several anti-G Abs and expressed as percentages of nuclei in syncytia relative to the control value (relative percentage of nuclei in syncytia).

The relative percentage of nuclei in syncytia obtained in presence of mice Abs to frg11 (aa 56–110), one of the fusion domains of the protein G of VHSV (Estepa et al., 2001; Nuñez et al., 1998), was reduced more than four-fold compared to the number of nuclei in syncytia found in the absence of Abs (from 100 to ~25%). Similar results were obtained with Abs to p2 (domain of frg11 implicated in fusion) but no reduction in the relative percentages were found with



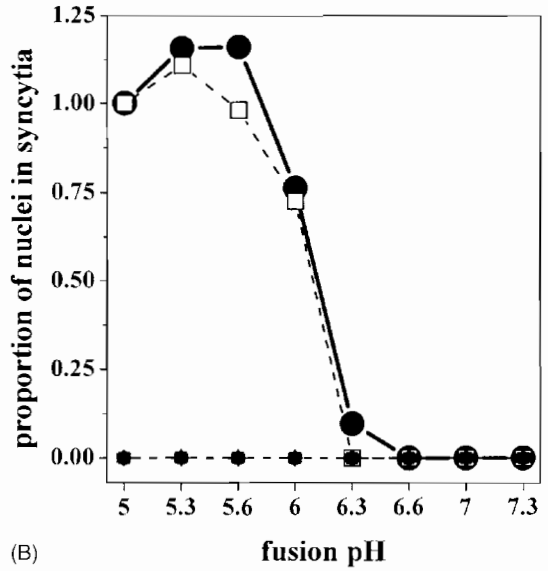


Fig. 3. Membrane expression of pG mutants in EPC cells transfected with pMCV1.4-G (A) and relative percentage of nuclei in syncytia obtained at different pHs (B). (A) Two days after transfection, the EPC cell monolayers were stained with rabbit-anti-G Abs and FITC labelled goat anti-rabbit IgG FITC (GAR-FITC). Thin lines, transfected cells stained only with GAR-FITC. Wide lines, transfected cells stained with anti-pG and GAR-FITC. The P79A mutant, included as a control, showed a similar profile than G98A. Non-transfected EPC cells did not show any difference of staining in the presence or absence of anti-G Abs. (B) Two days after transfection, the cell monolayers were changed to different pHs during 15 min and then 2 h at pH 7.6. After Giemsa staining, the number of nuclei in syncytia were counted ($n = 400$ per well). The results were calculated as % of nuclei in syncytia. The relative percentage of nuclei in syncytia (relative to pH 5) were then calculated by the formula, % of nuclei in syncytia at different pH's/% of nuclei in syncytia at pH 5. The numbers are the average of two experiments: (●) wt.; (□) P79A; (◆) P86A; (▲) G98A; (■) P86AG98A.

Abs to other segments of protein G, confirming results published before (Estepa et al., 2001).

Neutralising monoclonal Abs (MAbs) C10 and 3FIA12 reduced to near 0% the relative percentage of nuclei in syncytia (Fig. 4A). Similar results were obtained with MAb IP1H3 which reacted against a linear epitope but not by anti-G MAb 110.

Ascites from mice immunised with concentrated VHSV and saponin with neutralisation titre of ~1000 reduced to 15–40% the relative percentage of nuclei in

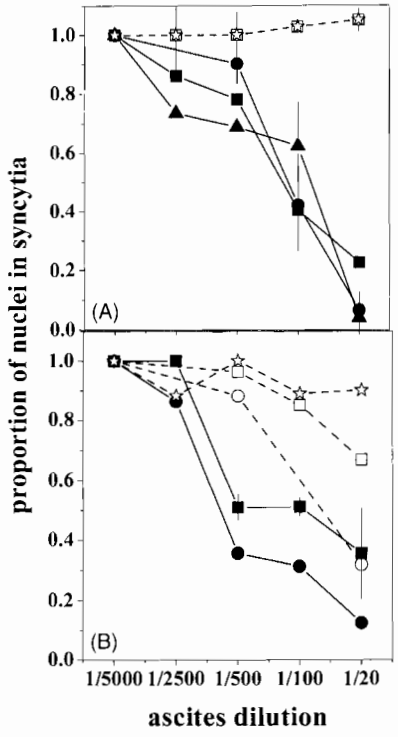


Fig. 4. Inhibition of low pH-dependent syncytia formation in G gene transfected EPC cells by anti-G MAbs (A) and anti-VHSV or anti-G polyclonal Abs (B) made in mice. Transfected EPC monolayers were assayed for antibody inhibition of low pH-dependent fusion as described. After Giemsa staining, the number of nuclei in syncytia were counted ($n = 400$ per well). Percentage of nuclei in syncytia in the absence of Abs (control) were $25.3 \pm 6.2\%$ ($n = 3$). Results are expressed as the proportion of nuclei in syncytia relative to the control by the formula: number of nuclei in syncytia in the presence of Abs/number of nuclei in syncytia in the absence of Abs. Averages and standard deviations from two determinations are represented. (A) (☆) Anti-N VHSV MAb 2C9 (Sanz and Coll, 1992); (●) MAb C10, neutralising anti-VHSV (MAb resistant mutants mapping at positions 140 and 433 of G) (Bearzotti et al., 1995; Gaudin et al., 1999a); (■) MAb 2 F1A12, neutralising anti-VHSV (MAh resistant mutants mapping at position 253 of G) (Lorenzen, Personal communication); (▲) MAb IP1H3, anti-VHSV G (mapped by pepscan between positions 399–413 of pG) (Fernandez-Alonso et al., 1999b); (□) MAb 110, anti-VHSV G (mapped by pepscan between positions 139–153 of G). (B) (☆) Ascites from mice immunised with an irrelevant antigen; (●, ■) ascites from mice immunised with VHSV adjuvanted in saponin (neutralisation titre ~1000); (○, □) ascites from mice immunised with purified G adjuvanted in Freund's (neutralisation titre ~100).

syncytia, while ascites from mice immunised with purified G and Freund's adjuvant with neutralisation titre of ~100 reduced to 30–40% the relative percentage of nuclei in syncytia (Fig. 4B).

3.4. Formation of syncytia in the presence of trout Abs

Fig. 5 shows the relative percentage of nuclei in syncytia obtained in the presence of sera from VHSV immunised trout. Sera from immunised trout with neutralisation titres of ~100,000 or ~2000, reduced to about 40% the relative percentage of nuclei in syncytia obtained in the absence of Abs (100%). Sera from immunised trout with neutralisation titres of ~100 reduced to about 60% the relative percentage of nuclei in syncytia obtained in the absence of Abs. In contrast, sera from healthy non-immunised trout did

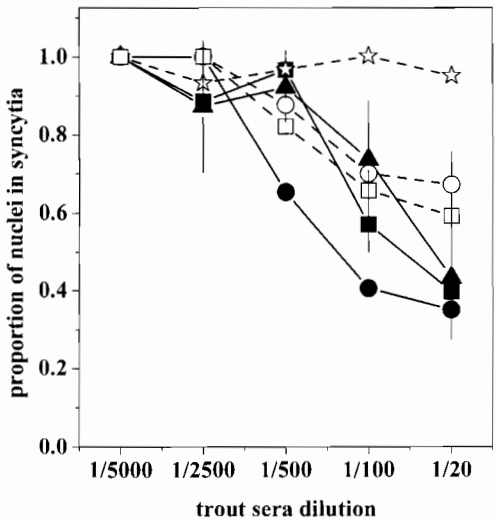


Fig. 5. Inhibition of low pH-dependent syncytia formation in G gene transfected EPC cells by trout serum. Transfected EPC cell monolayers were assayed for antibody inhibition of low pH-dependent fusion as described and results expressed as in Fig. 4: (☆) serum from one healthy non-immunised trout (similar results were obtained by 14 other non-immunised trout sera); (■, □, ▲) serum from trout with neutralisation titres of ~100,000 (kindly provided by Dr. Kinkelin of Jouy-in-Josas, France and Dr. Lorenzen of Aarhus, Denmark); (●) serum from trout (trout 3) immunised with VHSV and saponin with neutralisation titre of ~2000; (○) average and standard deviations from two serum from trout (trouts 1 and 2) immunised with VHSV + G and Freund's with neutralisation titres of ~100.

not reduce the relative percentage of nuclei in syncytia obtained in the absence of Abs.

4. Discussion

The characterisation of the pH-dependent syncytia formation of G gene transfected EPC cells has been described. Conditions for the highest efficiency and reproducibility of transfection of fish cells together with a cell monolayer stable to exposure to pH 6, are both needed for successful syncytia formation. To transfect EPC cell monolayers with the highest and most reproducible efficiency, fugene was preferred among 10 transfection reagents used because it induced the highest expression of reporter genes and it was the easiest to manipulate (Lopez et al., 2001). After complexing the fugene with the plasmid, the mixture is simply added to the EPC cell monolayers in the presence of its cell culture medium containing serum. Next day, the syncytia forming assay can be performed and results can be analysed 1 h later.

When ~20% of the EPC cells present in the monolayer were transfected, the number of nuclei in syncytia was ~12% of the nuclei present in the monolayer. That result suggests that a relatively high percentage of the G transfected cells become fused with nearby cells at the conditions used. Improvement of transfection efficiencies with the longer CMV promoter present in pMCV1.4 (Rocha et al., in press), can be incorporated to the assay thus increasing the number and size of the syncytia to facilitate subsequent analysis.

That the formation of syncytia was G-dependent was shown by detection of the protein G in the membrane of transfected cells by FACS, by the inhibition of syncytia formation when a mutated form of the G gene (single or double mutants in P86A and/or G98A) were used for transfection and by the inhibition of syncytia formation by both specific polyclonal and MAbs.

In the presence of mice ascites or trout serum from VHSV immunised animals, the number of syncytia was reduced in contrast to data obtained in the presence of control ascites or serum. Although mice ascites, neutralising MAbs and trout serum with the highest neutralisation titres showed also the highest anti-fusion titres, it was difficult to reduce the number of syncytia to 0% even when using high titered Abs directed against frg11 (one of the domains of protein G involved in fusion) (Estepa et al., 2001). That might be explained because the rapid change of conformation in the G protein induced by the low pH causes immediate fusion and inserts the implicated domains in the cellular membranes, thus avoiding the binding of some of the Ab molecules to those protein G domains.

Because a syncytia formation assay, such as the one described here, could be used to develop an assay for trout anti-fusion Abs without using virus, it could be an alternative method to estimate anti-VHSV Abs in those countries with no previous history of rhabdoviral infections. Knowledge of the immunological rhabdoviral state of salmonid populations by using this alternative method would be useful to assess the dangers of moving live fish, one of the most important ways to spread rhabdoviral diseases (DeKinkelin et al., 1995; Lorenzen et al., 1999, 2000). However, to be used as an anti-fusion trout Ab assay, a greater number of trout sera must be tested.

Detection of anti-VHSV fusion Abs might be also useful in conjunction with other methods that detect neutralising or protein G binding Abs, to help to further dissect the complex trout antibody response to VHSV infections or to monitor immunological responses during vaccine development.

Acknowledgements

Thanks are due to J.P. Coll for typing this manuscript. This work was supported by INIA projects RTA03-217, OT02-003 and SC00046, Spain.

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