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Antibody response to a fragment of the protein G of VHS rhabdovirus in immunised trout

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Abstract

A fragment (called frg#11, amino acids, aa 56-110) of the protein G (pG) of viral haemorrhagic septicaemia virus (VHSV) was designed after previous results showed it to be recognised by ~40% of the trout immunised to VHSV [Dis. Aquat. Organ. 34 (1999) 167]. frg#11 was then cloned, expressed, purified and used to study the production of antibodies to its epitopes in trout immunised to VHSV. Anti-frg#11 trout antibodies could be detected in serum from individual trout surviving VHSV exposure, immunised by injection with purified VHSV or DNA-immunised with its pG gene whereas it was not detected in non-infected and non-immunised trout. The trout serum antibodies which reacted more strongly by ELISA using solid-phase frg#11 (continuous or linear epitopes on the sequence of the pG) had the lowest VHSV-neutralising activity (epitopes which are pG conformation-dependent). Because antibodies recognising continuous as well as conformation-dependent epitopes of the pG seem to be involved in protective trout immunological responses to VHSV, the estimation of anti-frg#11 antibodies could help to the dissection of the complex trout antibody response to VHSV infections. In addition, these preliminary results suggest that the determination of anti-frg#11 antibodies might also be used to complement in vitro viral neutralising assays which seem to be restricted to pG conformation-dependent epitopes.

Keywords: Salmonid rhabdovirus; VHSV; Linear epitopes; Trout antibodies; Protein G; Recombinant fragment

1. Introduction

Despite the considerable advances obtained in the last years, the trout humoral immunological response to viral haemorrhagic septicaemia virus (VHSV) infection is still a matter of study (Lorenzen et al., 1993, 1999). For instance, it is not clear the relative importance in the protection against the disease of antibodies directed against continuous (linear) and conformation-dependent protein G (pG) epitopes. Thus, in vitro VHSV neutralising antibodies do not always correlate with its protection properties in vivo (LeFrancois, 1984; Lorenzen et al., 1999, 1990), some non-neutralising monoclonal antibodies directed toward linear epitopes (western blot positive under reduced conditions) provided protection against VHSV by passive immunisation (Lorenzen et al., 1990) and sera from trout immunised to VHSV with high neutralisation titres have none or lower titres to linear epitopes (Fernandez-Alonso et al., 1999b). Furthermore, non-neutralising antibodies (those directed mostly towards linear or continuous epitopes) tend to persist longer after VHSV infection than do neutralising antibodies (Lorenzo et al., 1995; Enzman and

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Konrad, 1993). All the above-mentioned results suggest the importance that antibodies directed to continuous or linear epitopes might play in the trout protective responses in this disease. Detection of anti-VHSV antibodies directed to linear epitopes might be useful in conjunction with methods that detect conformation-dependent neutralising antibodies on the pG to clarify their role in the trout responses to VHSV.

To follow up successful immunisation to VHSV during vaccination attempts, methods for estimating putative-protective pG-specific trout antibodies to VHSV are primarily based on in vitro neutralisation assays (Jorgensen et al., 1991; Lorenzo et al., 1996) or binding to VHSV-captured ELISAs (Olesen et al., 1991; Sanz and Coll, 1992a). The virus neutralisation methods are not only time-consuming, labour-intensive and require sterile conditions but are also restricted to the detection of neutralising antibodies which in the case of VHSV are pG conformation-dependent antibodies (Fernandez-Alonso et al., 1999b,c; Lorenzen et al., 1993, 1999). The binding to VHSV-captured ELISA method was able to detect trout antibodies to both conformational and linear epitopes in the pG, however it suffered from high backgrounds and some false positives (Olesen et al., 1991). Our previous attempts to detect trout anti-pG VHSV antibodies by ELISA employed purified VHSV as solid-phase but had high background, low sensitivity and required the preparation of large amounts of purified VHSV (Estepa et al., 1994). To increase sensitivity and/or decrease background, we tried to increase the number of G epitopes per well by using linearized recombinant G4 (amino acid, aa 9-443) synthesised in yeast after destroying the intermolecular disulphide bonds of its inclusion bodies (Estepa and Coll, 1996a; Estepa et al., 1994). However, due to the difficulties in the production and purification of G4, we searched for an alternative pG antigen. As a result we report here the use of a shorter amino-terminal fragment of pG called frg#11 (aa 56-110), implicated in viral fusion (Estepa et al., 2001) which contains a large proportion of the linear epitopes recognised by individual immunised trout (Fernandez-Alonso et al., 1999c). In this report, we show that the ELISA made with solid-phase frg#11 is sufficient to detect some of the anti-VHSV antibodies directed to linear epitopes with high sensitivity and low background. The trout serum antibodies which reacted more strongly by ELISA using solid-phase frg#11 (continuous or linear epitopes on the pG) had the lowest VHSV-neutralising activity (conformation-dependent epitopes on the pG). While this assay may have some diagnostic potential its main utility has been in the follow up of DNA immunisation attempts (Fernandez-Alonso et al., 2001) that confirmed the predictive value of the level of anti-frg#11 antibodies on the resistance to VHSV challenge. It could also help in furthering the understanding of the complex trout antibody response against VHSV by estimating the response to some linear epitopes of its pG.

2. Materials and methods

2.1. VHSV virus

VHSV 07.71, isolated in France (LeBerre et al., 1977) from rainbow trout *Oncorhynchus mykiss* (Walbaum) was used throughout the experiments. The VHSV was grown using epithelial papullosum cyprini (EPC) cells in cell culture medium as described previously (Basurco et al., 1991). VHSV was concentrated from infected EPC supernatants using 7% polyethylene glycol (PEG) 6000 in 2.3% NaCl, pH 7.8 as previously described (Basurco et al., 1991).

2.2. VHSV microneutralisation assay

About 10^3 $TCID_{50}$ per millilitre of VHSV 07.71 were incubated overnight at 4 °C with serial dilutions of trout serum containing anti-VHSV antibodies in cell culture medium. To assay for trout neutralising antibodies, 10% of fresh non-immunised trout serum was added to the virus/antibody mixtures as a source of trout complement. 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 with cell culture medium, filled with 100 μl/well with culture medium and incubated overnight at 14 °C. The VHSV infected EPC monolayers were fixed during 10 min in cold methanol and dried. To detect the N antigen, the MAb 2C9 diluted 1000-fold in dilution buffer (130 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.24 mM merthiolate, 5 g of Tween-20/l, 50 mg of phenol red/l, pH 6.8)

was added to the wells (100 μl/well) and incubated for 1 h. After washing with distilled water, 100 μl of peroxidase-labelled anti-mouse IgG (Nordic, Tilburg, The Netherlands) were added per well and incubation continued for 30 min. After three washings with distilled water, 50 μl of 1 mg/ml per well of diaminobenzidine (DAB) (Sigma) in the appropriate buffer was added, and the reaction allowed to proceed until brown foci were detected by inspection with an inverted microscope in the controls containing no antibodies. Neutralisation titre was defined as the reciprocal of the maximal dilution that reduced DAB positive foci to its minimum number, usually 1–2 foci per well (Lorenzo et al., 1996).

2.3. Cloning and yeast expression of G4

The recombinant G4 (aa 9-443) was derived from the VHSV G gene as described (Estepa et al., 1994). Briefly the cDNA from VHSV was amplified by PCR and cloned into the pEGT110 plasmid (Eurogentec, Liege, Belgium) under the alcohol dehydrogenase/galactose phosphate dehydrogenase (ADH/GAPDH) hybrid promoter. *S. cerevisiae* strain DCO4 (leu) was used as the yeast expression strain after electroporation of the G4-pEGT110 construct. Recombinant protein yeast extracts were centrifuged to obtain inclusion bodies with about 6% protein being of G4 as determined by (polyacrylamide gel) PAGE and densitometer measurements. Inclusion bodies were heated to 100 °C in the presence of 1 M β-mercaptoethanol and 8 M guanidinium chloride for 4 h. Solubilized inclusion bodies were separated by PAGE and the 45 KDa band electroeluted. Purity of these preparations was about 48% as determined by PAGE and densitometry using the Scion Image v4.02 program (Scion, Frederick, MD, USA).

2.4. Cloning of frg#11

The DNA sequence corresponding to aa 56-110 of the pG of VHSV (Thiry et al., 1991) was amplified by the polymerase chain reaction from VHSV cDNA using gene specific primers. The amplimer 1 forward or sense primer (5′-GTCGGATCC**ATG**CCGATTCGACCA) hybridised with the cDNA region corresponding to the N-terminus of the glycoprotein and contained a *Bam*HI restriction site and an initiating ATG. The reverse or antisense primer 2 (5′-CGAGAATTCTCAACAGG**TGA**CTCG) hybridised with the cDNA region corresponding to the C-terminus and contained an *Eco*RI restriction site and a stop TGA. The PCR-generated DNA were recovered from the agarose after gel electrophoresis, digested with *Bam*HI and *Eco*RI, and cloned into the linearized pRSETa plasmid (Invitrogen, San Diego, CA). The construction was then used to transform the *E. coli* DH5α strain according to the Invitrogen protocol.

2.5. Bacterial expression of recombinant frg#11

For expression of the cloned frg#11, *E. coli* BL21 DE3 cells were transformed with the pRSETA-frg#11 plasmid from the DH5α strain. The *E. coli* were grown in 1 l of LB medium supplemented with ampicillin at 37 °C. When the culture reached an optical density at 600 nm of 0.7, the temperature was down shifted to 28 °C and 100 mM IPTG was added to induce expression of frg#11. The bacteria cells were grown for 6 h before adding a second 100 mM IPTG and the culture was maintained overnight at 28 °C. The procedure was scaled up to 15 l with the aid of a fermentor (Inbiotec, León, Spain). Finally, the cells were pelleted by centrifugation and subsequently treated with 30 ml of lysis buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 6 M guanidine HCl, pH 7.8). The lysate was submitted to 25 sonication bursts and then centrifuged 20,000 × *g* for 30 min. The supernatant was collected and applied to a Ni affinity column (ProBond™, Invitrogen) previously equilibrated with binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 8 M urea, pH 7.8). Washing buffer at pH 6 was passed through the column before the elution buffer at pH 4 (composition of buffers as provided by manufacturer). Concentration of the protein in the collected fractions was monitored by absorbance at 280 nm. Peak fractions were pooled and dialysed against 20 mM Tris at pH 4 to increase solubility. To increase the purity of the pooled fractions these were then chromatographed by Sephadex G-100 in 20 mM Tris at pH 4. Concentrations and purity were determined by either absorbance at 590 nm (Bradford) and the densitometry of Coomassie-blue stained protein bands on 15% PAGEs of the protein solutions using a standard curve generated with BSA.

2.6. Characterisation of frg#11 by ELISA and immunoblotting

Confirmation of frg#11 expression was performed by ELISA using polyclonal antibodies (Estepa and Coll, 1996b) obtained in mice against affinity purified glycoprotein G from VHSV (Perez et al., 1998). Serial dilutions of frg#11 in 100 μl water were pipetted per well (Polysorp, Nunc) and allowed to dry overnight at 37 °C. Anti-G mice antibodies diluted 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) were added to the plates and incubated for 1 h. After washing with distilled water, 300-fold diluted horseradish peroxidase-conjugated goat-anti-mouse antibody (Sigma, St. Louis, MO) was added. The colour reaction was developed by adding 1 mg/ml *o*-phenylendiamine in citrate buffer containing 3 mM H_2O_2. Optical density was measured by using dual wavelength measurements at 492–620 nm in a Labsystems ELISA reader. The optical density at 620 nm was used to correct for individual differences between wells as recommended by the manufacturer.

The ability of mice or trout anti-frg#11 antibodies to react with recombinant bacterial (frg#11) or yeast (G4) lysates was tested by western-blot analysis. SDS-15% acrylamide gels were loaded with induced-recombinant bacterial or yeast lysates in buffer containing 2-mercaptoethanol. The proteins in the gel were transferred to nitrocellulose membranes (BioRad, Richmond, VI). The membranes were blocked with 2% dry milk, 0.05% Tween-20 and 0.3% rabbit serum in PBS and cut into 3 mm wide strips. Each strip was incubated with anti-VHSV or anti-frg#11 mice polyclonal antibodies before incubation with the peroxidase-conjugated rabbit anti-mouse antibody and developed with DAB staining.

2.7. Production of anti-pG and anti-frg#11 polyclonal antibodies in mice

To characterise recombinant frg#11, each female BALB/c mouse were first injected with 20 μg of concanavalin A purified pG (Perez et al., 1998), 1% formalin treated for 1 h treated with purified pG or purified recombinant frg#11, in Freund's complete adjuvant. Then four monthly injections with the same antigens in Freund's incomplete adjuvant were carried out. To obtain ∼40 ml of pooled diluted ascites, 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 through a 3 × 10 cm Sepharose column (Pharmacia) with bound *E. coli* protein extract (∼10 mg/ml) and rabbit serum (∼10 mg/ml) to adsorb background antibodies and concentrated with 40% ammonium sulphate. The concentrate was 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.

2.8. Production of anti-VHSV polyclonal antibodies 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 of two trout per group. One group, trouts 1 and 2 were immunised with Freund's + concentrated VHSV and G4 and another group with saponine + concentrated VHSV. Trout were injected intraperitoneally four times during 3 months with 400 μl of the VHSV + G4 antigen preparations. To each trout 30 μg of heat killed (37 °C, 30 min) PEG-concentrated VHSV + 30 μg of recombinant G4 were injected diluted 1:1 with complete Freund's adjuvant during the first injection and the rest of injections were diluted 1:1 with incomplete Freund's adjuvant. Neutralisation titres of trout sera 1 and 2 were of ∼100. Two other trout, trouts 3 and 4, were injected with concentrated VHSV in saponine. Concentrated VHSV (30 μg) was sonicated in the presence of 30 μg of saponin (Superfos, Quil A), 620 μg of cholesterol and 6 μg of phosphatidylcholine (Larsson et al., 1993; Morein et al., 1984) in 1 ml of distilled water. Neutralisation titres of trout sera 3 and 4 were of ∼2000. Two other trout sera with neutralisation titres ∼100,000 were kindly provided by Dr. De Kinkelin (France) and Dr. Lorenzen (Denmark).

The plasmid G3-pcDNAI/Amp coding for the pG gene of VHSV (Fernandez-Alonso et al., 1999a) was used to induce anti-G antibodies in a large number of fingerling trout. The plasmid was transferred and multiplied in the *E. coli* strain Top10F. Large amounts

of plasmids were prepared from recombinant *E. coli* pellets using the Wizard plus Megaprep DNA purification system (Promega, Madison, WI). To immunize fingerling trout, plasmid solutions were adjusted to 1 mg/ml of total DNA (absorbance at 260 nm) and 10 μg of plasmid per millilitre were sonicated with 5–10 trout of 20–50 g of body weight in 200 ml of water as described recently in detail (Fernandez-Alonso, 2000; Fernandez-Alonso et al., 2001). Fingerling trout were used to donate serum 1 month after immunisation.

To generate trout survivors of VHSV infections, about 400 trout weighting between 0.5 and 2 g at the time of infection were infected by immersion in VHSV 07.71 (Basurco and Coll, 1992). The trout surviving the infection were challenged 1–3 months later with VHSV 07.71. After 1 month, survivor trout showed no sign of VHS and were bled to donate serum 2–4 months after the last VHSV challenge (100–200 g per trout).

As control trout serum, 15 healthy non-infected trout (500 g of body weight) were obtained from a farm with no history of VHSV infections (Escuela de Montes, Madrid, Spain) and used either single or pooled according to the experiments (control pooled trout serum).

2.9. Anti-pG ELISA assays in trout sera

To assay for trout anti-viral G antibodies in trout sera, polystyrene plates of 96-wells (Dynatech, Plochingen, W. Germany) were coated with 2 μg of purified G4 or frg#11 per well in 100 μl of distilled water, overnight at 37 °C. To reduce the background, the coated wells in the plates were blocked overnight at 4 °C with 100 μl per well of dilution buffer (as described above) and washed before performing the ELISA. The trout antibodies were serially diluted from 30 to 810-fold in dilution buffer (see before). The plates were incubated for 60 min at room temperature with 100 μl/well of diluted trout sera. After washing, they were incubated for 30 min with anti-trout immunoglobulin monoclonal antibody (MAb) 1G7 (Sanchez et al., 1991). Other details were as described above or before (Sanz and Coll, 1992b).

3. Results

Because ∼40% of individual responses in sera from several trout immunised with VHSV (Fernandez-Alonso et al., 1999c), were obtained to the peptides from the pG region around aa 100, and this region has been shown to be related to VHSV fusion (Nuñez et al., 1998; Estepa et al., 2001), frg#11 (aa 56-110) was selected for further experiments.

frg#11 was cloned from VHSV RNA, expressed in *E. coli* and purified as described in methods. The nucleotide sequences corresponding to frg#11 were present in the recombinant plasmid insert as demonstrated by DNA sequencing of the insert in both directions (not shown). To confirm that the fragment cloned corresponded to the pG sequence, its amino acid (aa) composition was also determined. Purified frg#11 showed an aa composition after hydrolysis very similar to that expected by its deduced aa composition (not shown). The presence of aa sequences of the pG of VHSV in the purified frg#11 was further confirmed by using anti-G protein and anti-frg#11 antibodies raised in mice. Thus specific anti-purified G mice antibodies made with purified native or cross-linked pG antigens which recognised solid-phase purified VHSV (not shown) and purified native pG also recognised frg#11 (Fig. 1).

The expressed reduced purified recombinant frg#11 migrated as a band in PAGE with an apparent molecular weight of about 10 KDa (expected 10,355 Da) (Fig. 2A, V + I at 6 and 24 h), which was absent in uninduced recombinant bacteria containing the frg#11 sequence (Fig. 2A, V + I at 0 h) or in induced bacteria without the insert (Fig. 2A, V at 0, 6 and 24 h). This same band was the only one recognised by anti-frg#11 antibodies in immunoblotting of recombinant bacteria lysates (not shown). It was estimated by densitometry of the PAGE Coomassie-blue stained bands that frg#11 accounted for 16.3% of the total bacterial protein and it could be obtained with 88.9% of purity after Ni affinity and Sephadex G-100 chromatographies (Fig. 2B, frg#11).

The capacity to detect anti-pG VHSV antibodies from trout immunised with VHSV + G4 (trout 1) was compared by using ELISA plates coated with purified recombinant G4 (aa 9-443) or frg#11 (aa 56-110) antigenic preparations. Fig. 2C shows that both antigenic preparations produced about the same ELISA absorbance profiles when used as solid-phase up to 0.8 μg/well. Increasing the amount of antigens in the solid-phase also increased the absorbance to frg#11

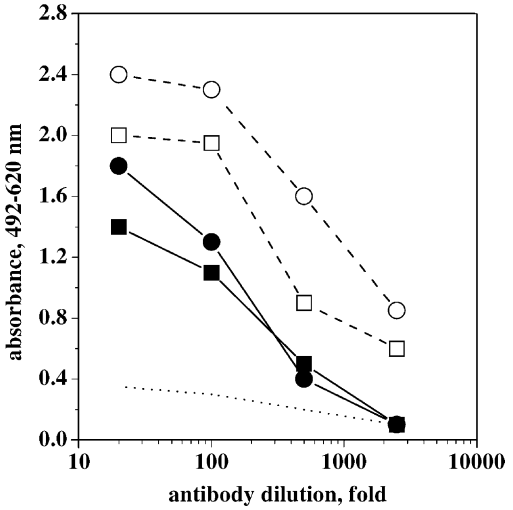

Fig. 1. Recognition of solid-phase frg#11 or native pG by murine antibodies against native or fixed pG from VHSV. Mice antibodies against ConA-affinity purified pG (Perez et al., 1998) either native (●, ○) or after fixing with formaldehyde (■, □) were used to recognise solid-phase frg#11 (black points) or native pG (open points) (2 μg per well). One of two experiments is represented. (● — ●): binding of anti-native pG antibodies to solid-phase frg#11; (■ — ■): binding of anti-fixed pG antibodies to solid-phase frg#11; (○ – – – ○): binding of anti-native pG antibodies to solid-phase native pG; (□ – – – □): binding of anti-fixed pG antibodies to solid-phase native pG; (– – –): binding of anti-native or anti-fixed G antibodies to solid-phase with no protein. Binding of serum obtained from non-immunised mice gave background values with solid-phase frg#11 or pG (not shown).

but not to G4, most likely due to the lower 48% purification of G4 (Fig. 2B, G4) compared with the higher 88.9% purification of frg#11. Values as high as ∼2.5 absorbance units could be obtained using trout sera 30-fold diluted and 6 μg of frg#11 per well but the best compromise between level of absorbance and amount of frg#11 in the solid-phase could be obtained at 2 μg/well. Background absorbances obtained with control pooled trout serum were lower than 0.05, even when 6 μg/well of frg#11 were used, suggesting that antibodies to frg#11 could be assayed in trout sera with a high sensitivity. The lower ELISA absorbances obtained when a ∼5-fold less purified preparation of frg#11 were used to coat the wells suggest that purity of the antigen is also important to obtain the best ELISA values and that trout antibodies do not bind non-specifically to any bacterial protein that could be still present in higher purified frg#11 (Fig. 2C).

We then proceeded to assay individual trout sera with different neutralisation titres by ELISA using a fixed concentration of solid-phase frg#11 (2 μg/well). Fig. 3 shows the decrease in the absorbance obtained by the reaction of increasing dilutions of different trout sera with solid-phase frg#11. Sera from trouts 1 and 2 immunised by injection with VHSV + G4 + Freund's adjuvant with the lowest neutralisation titres (∼100) have the highest absorbance values at 30-fold dilutions (0.6–0.8 units). The variation of the profiles of absorbances with sera dilutions were very similar for the two sera. Sera from trouts 3 and 4 immunised by injection of VHSV + saponin adjuvant with ∼2000 neutralisation titres (Fig. 3) and sera with ∼100,000 neutralisation titres (not shown), have lower absorbance values at 30-fold dilutions (∼0.2 units), whereas sera from pooled control trout serum at 30-fold dilutions have only about 0.02 absorbance values (background values). Therefore, the low background values obtained at 30-fold dilutions of control pooled trout serum would allow to assay trout sera at this low dilution thus increasing detectability of the low levels of anti-frg#11 antibodies which might be present in some trout serum. The trout serum which had the highest neutralisation titres showed the lowest anti-frg#11 antibody titres, confirming the observations made earlier with anti-G4 antibodies (Fernandez-Alonso et al., 1999b).

To compare the sensitivity to detect continuous or linear antibodies to the pG of VHSV by using either G4 or frg#11 in solid-phase ELISA, different individual sera from trout immunised with a plasmid codifying the G protein of VHSV were assayed by both methods. On the average most G immunised trout sera yielded a higher OD value when estimated by solid-phase frg#11 (0.61 ± 0.32) than by G4 (0.38 ± 0.35) (Fig. 4).

In a preliminary evaluation of the possible use of frg#11 to detect trout antibodies to the pG of VHSV, we obtained higher numbers of individual sera from fingerling trout survivors of two consecutive VHSV infections, immunised with purified VHSV and immunised with a plasmid codifying the G gene. Table 1 shows that in contrast to the averaged background values obtained with the non-injected or control plasmid (pcDNAI/Amp) injected trout (1.1 ± 0.5, $n = 15$

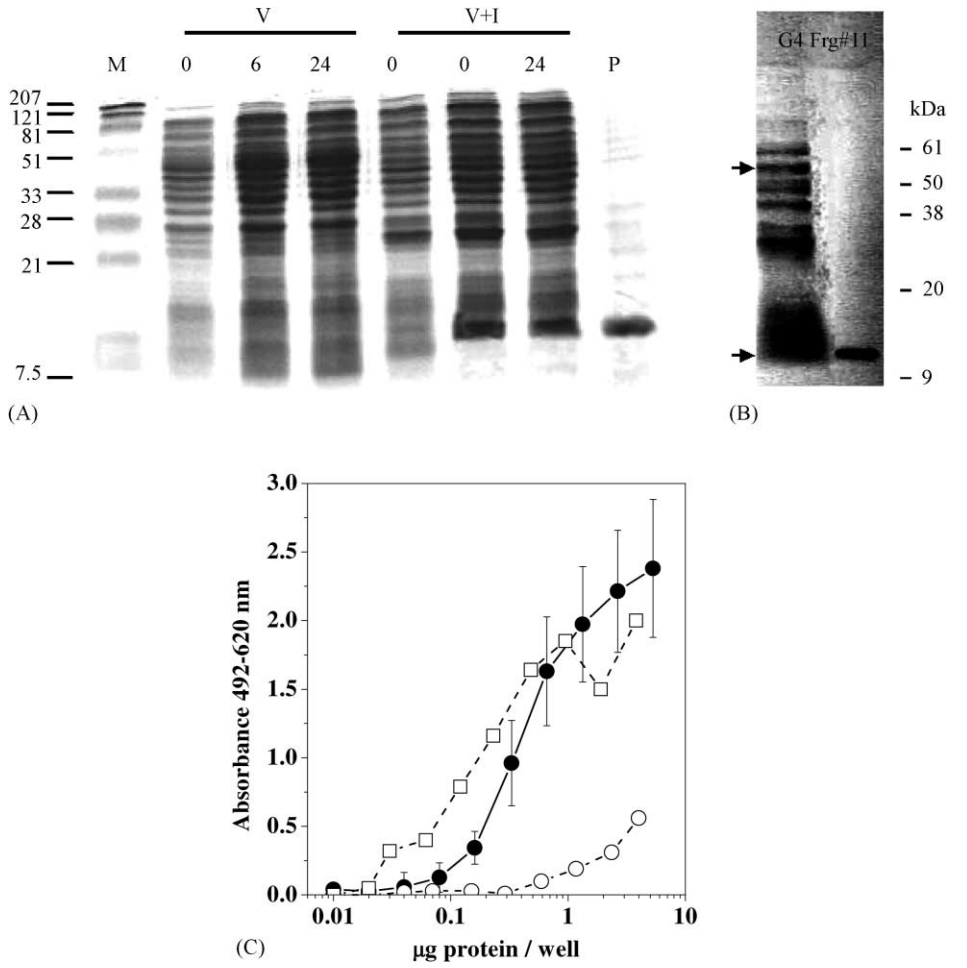

Fig. 2. Results of the expression of frg#11 shown by PAGE (A). Comparison of purified G4 and frg#11 by PAGE (B) and relationship between the amount of solid-phase VHSV antigen and the absorbance obtained with VHSV immunised trout serum (C). (A) Grown cultures of *E. coli* carrying pRSETA (vector, V) or pRSETA-11 plasmids (vector plus frg#11 insert, V + I) were induced with 100 mM IPTG. Samples were collected at 0, 6 and 24 h post induction, analysed by 15% PAGE and Coomassie-blue stained to visualise the proteins. Molecular weight markers identified with the numbers to the left (M). frg#11 purified by Ni affinity chromatography (P). (B) PAGE of the electroeluted purified G4 and Ni affinity plus Sephadex chromatographies purified frg#11 used to coat the ELISA plates. Low molecular weight peptides most probably due to proteolysis of the yeast protein are detected in the G4 preparations. Numbers to the right are the molecular weight markers in kilo Dalton. Arrows to the left indicate the specific bands which reacted by immunoblotting with anti-VHSV polyclonal antibodies (not shown). (C) Serum from trout 1 diluted 30-fold was used to perform the assay. Averages and standard deviations from three experiments are represented for purified frg#11, the results of one experiment are represented for the other antigens. (● — ●): frg#11 purified by Ni affinity plus Sephadex chromatographies to 88.9% of purity; (○ – – – ○): frg#11 before purification of the bacterial extract with 16.3% of purity; (□ – – – □): G4 purified by PAGE to 48% of purity. Absorbances lower than 0.15 were obtained by using solid-phase purified frg#11 with control pooled trout serum.

or $0.8 \pm 0.2, n = 12$), averaged values obtained for sera from trout immunised by injection with VHSV, trout survivors or trout immunised by immersion with the G-plasmid were 4.0 ± 2.7 ($n = 8$), 1.9 ± 0.9 ($n = 10$) or 2.2 ± 0.4 ($n = 24$)-fold higher, respectively. In parallel experiments, ELISA made by using G4 as solid-phase, no differentiation of average absorbances could be made among the same sera (not shown).

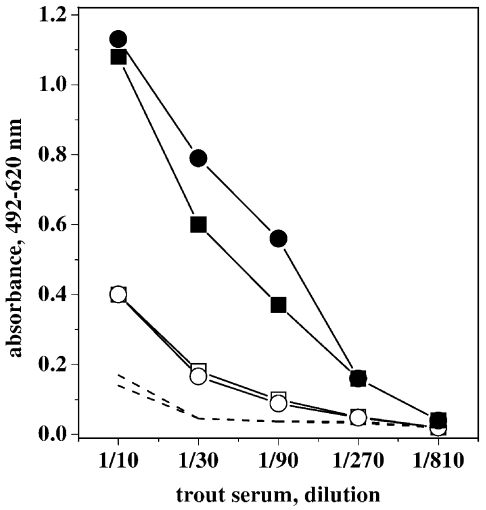

Fig. 3. Trout serum titration on solid-phase frg#11. Trout serum immunised to VHSV with different neutralisation titres were assayed for frg#11 (2 μg per well) reactive antibodies. One of two experiments is represented. (● — ●): serum from trout 1 (neutralisation titre of ∼100); (■ — ■): serum from trout 2 (neutralisation titre of ∼100); (○ — ○): serum from trout 3 (neutralisation titre of ∼2000); (□ — □): serum from trout 4 (neutralisation titre of ∼2000); (– – –): control pooled serum obtained from healthy uninfected trout assayed in duplicate.

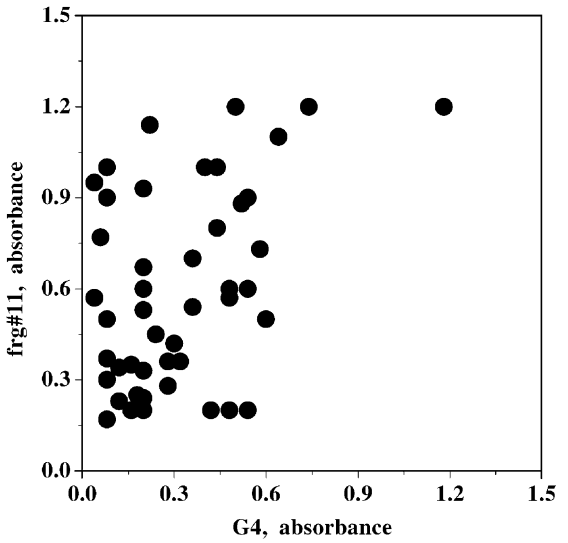

Fig. 4. Relation between the ELISA absorbance values obtained by the reaction of sera from trout immunised with the G3-pcDNAI/Amp plasmid and solid-phase G4 and frg#11. Plates were coated with 2 μg of either G4 (G4 absorbance) or frg#11 (frg#11 absorbance). Sera were obtained from 46 trout immunised with G3-pcDNAI/Amp. The sera were analysed by ELISA using 3-fold dilutions from 1/10 to 1/270. The dilution at 1/30 was employed for the comparison because most absorbances were distributed between 0.3 to 1.2 absorbance units.

Table 1
Estimation of anti-frg#11 antibodies in serum from trout immunised against VHSV[a]

Trout	Relative absorbance
Injected with VHSV	**4.0 ± 2.7 (8)**
VHSV survivors	**1.9 ± 0.9 (10)**
Immersion in G3-pcDNAI/Amp	**2.2 ± 0.4 (24)**
Injected with pcDNAI/Amp	0.8 ± 0.2 (12)
Non-injected	1.1 ± 0.51 (15)

[a] Plates were coated with 2 μg of frg#11 per well. The ELISA results were obtained with different dilutions of the trout serum as in Fig. 3. The dilution at 30-fold was selected as the best to compare the results for each sera, because most values were between 0.3 to 1.2 absorbances. The results are expressed as the absorbance relative to the absorbance obtained with pooled control trout serum obtained from 15 healthy non-injected trout, according to the formula, absorbance obtained with each trout serum/average of absorbance obtained with control pooled trout serum. Averages ± standard deviations are represented. The number in parenthesis is the number of trout sera assayed. The numbers in bold are significantly different from trout sera from either injected with pcDNAI/Amp or non-injected.

4. Discussion

Despite the different immunisation strategies with different forms of pG antigens and its variation in neutralisation titres, the profiles of individual trout serum reactivity with the pepscan of the pG were very similar (Fernandez-Alonso et al., 1999c). The continuous or linear epitopes situated around aa 100 (frg#11) contained the heptad repeats, the highest phosphatidylserine binding region of VHSV involved in fusion (Estepa and Coll, 1996a; Nuñez et al., 1998; Estepa et al., 2001) and some trout T-like epitopes (Lorenzo et al., 1995). Therefore, we have explored the possibility of using frg#11 as solid-phase to increase the epitopic density in ELISA and to be able to study the relation of anti-frg#11 antibodies to VHSV immunity.

Because trout serum non-specifically binds to many surfaces and/or proteins (Jorgensen et al., 1991; Olesen et al., 1991), it has been difficult to develop methods to detect specific trout anti-pG antibodies with high sensitivity by using plates coated with

purified VHSV or VHSV-captured by solid-phase MAbs. In addition, a low density of G epitopes per well and therefore a low sensitivity is to be expected by using purified VHSV to detect pG specific antibodies because most of the epitopes contained in VHSV belong to the majority N protein. An increase in the density of solid-phase pG epitopes is necessary if antibodies to them need to be detected with maximum sensitivity. One way to increase the pG epitope density is by using purified G from VHSV. However, the difficulties in obtaining large amounts of purified G made this option not feasible (Perez et al., 1998). An other alternative is to use purified recombinant G proteins. However, because recombinant proteins do not have a native conformation their use as solid-phase in ELISA will only detect antibodies directed to continuous, linear or non-native epitopes and not the conformational-dependent epitopes involved in virus neutralisation very often found in discontinuous epitopes (Lorenzen et al., 1999; Fernandez-Alonso et al., 1999b,c).

Because of the lower molecular weight of frg#11 (10,355 Da) when compared to the glycosilated pG (∼55,000 Da), a greater number of frg#11 molecules per surface unit could coat the solid-phase thus increasing the epitopic density 4–5-fold. Furthermore, because the frg#11 region is recognised by 40% of the sera from trout hyperimmunized with VHSV, ELISA using solid-phase frg#11 could detect a representative percentage of the trout antibody response to the continuous linear epitopes of VHSV even though some conformation could also be present in the frg#11 preparations. Confirming those expectations, the absorbances of 46 trout sera immunised with a G-containing plasmid obtained by using frg#11 (aa 56-110) as solid-phase were ∼2-fold greater than by using G4 (aa 9-443) (Fig. 4), despite the greater number of linear epitopes that should be present in the G4. However, higher absorbances could also mean that the same antibodies will bind more effectively than it would to the same epitope within the G4 context and not because more epitope is present. Further analysis of the trout, different antibody species present in each single sera will clarify these aspects.

The previously reported mapping of linear epitopes recognised by anti-VHSV trout antibodies demonstrated three main antigenic regions in the pG of VHSV (Fernandez-Alonso et al., 1999c) which mapped outside the mutant aa discontinuous positions identified by the sequencing of monoclonal antibody resistant (MAR) mutants (Bearzotti et al., 1995). Since MAR mutants, most probably map the neutralised epitopes, the use of linear epitopes as antigens for ELISA would detect antibodies other than the neutralising antibodies. The serum antibodies from VHSV immunized trout which reacted strongly by ELISA using solid-phase frg#11 have lower VHSV-neutralising activity (Fig. 3), confirming previous reported observations by ELISA using solid-phase G4 (Fernandez-Alonso et al., 1999b). Furthermore, none of the 46 sera from trout immunised with G3-pcDNAI/Amp have any detectable neutralisation titer (not shown). Thus it seems likely that sera from trout immunized with VHSV falls into three categories, those having high neutralisation and low binding antibody titres, those having low neutralisation and high binding antibody titres and those having both of them with low titres. Although these results should be regarded as preliminary because of the small numbers of fish examined, taken together with previous findings, they suggest the complementarity of the two types of humoral antibody response to immunize trout to VHSV.

From the practical point of view, production of recombinant *E. coli* frg#11 is more easily reproducible than recombinant yeast G4. Thus, production of recombinant yeast with G4 inclusion bodies (Estepa et al., 1994) and down stream purification of G4 are both time-consuming and complicated processes. Furthermore, despite the complex procedure to disrupt the G4 inclusion bodies and its purification by PAGE, only 48% of purity could be obtained with consistency. In contrast, growth of recombinant *E. coli* and IPTG induction of frg#11 expression can be done more easily and scaled up in a fermentor to 15 l. Subsequent purification by affinity and gel permeation columns can yield preparations with >90% of purity. The above-mentioned reasons together with the high sensitivity and low background obtained in ELISA using frg#11 made this recombinant fragment to be preferred to G4 to estimate linear antibody responses to the pG of VHSV.

The frg#11-based ELISA method to detect trout antibodies to continuous or linear epitopes of the G protein might be useful to recognise large number of samples from trout previously exposed to VHSV as a

complement to current methods to detect trout antibodies to conformation-dependent neutralising epitopes, as shown by the results obtained in this work (Table 1). However, the number of trout sera evaluated with this method in this work was still very limited and further assays to determine specificity should be performed (preliminary results showed that antibodies to infectious necrosis virus, IHNV or to infectious pancreatic necrosis virus, IPNV do not recognise frg#11). More rigorous assay validation is required before the results from this test can be used for diagnostic purposes, as this was not intended in the present work. However, this method might help to the dissection of the complex trout antibody response to VHSV infections and to explorations of its predictive value to the protection of candidate vaccines.

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