Mapping of linear antibody epitopes of the glycoprotein of VHSV, a salmonid rhabdovirus

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ABSTRACT: Antibody linear epitopes of the glycoprotein G (gpG) of the viral haemorrhagic septicaemia virus (VHSV), a rhabdovirus of salmonids, were mapped by pepscan using overlapping 15-mer peptides covering the entire gpG sequence and ELISA with polyclonal and monoclonal murine and polyclonal trout antibodies. Among the regions recognized in the pepscan by the polyclonal antibodies (PAbs) were the previously identified phosphatidylserine binding heptad-repeats (Estepa & Coll 1996; Virology 216:60–70) and leucocyte stimulating peptides (Lorenzo et al. 1995; Virology 212:348–355). Among 17 monoclonal antibodies (MAbs), only 2 non-neutralizing MAbs, I10 (aa 139–153) and IP1H3 (aa 399–413), could be mapped to specific peptides in the pepscan of the gpG. Mapping of these MAbs was confirmed by immunoblotting with recombinant proteins and/or other synthetic peptides covering those sequences. None of the neutralizing MAbs tested reacted with any of the gpG peptides. Previously mapped MAb resistant mutants in the gpG did not coincide with any of the linear epitopes defined by the pepscan strategy, suggesting the complementarity of the 2 methods for the identification of antibody recognition sites.

KEY WORDS: Epitopes · Glycoprotein · VHSV · Rhabdovirus

INTRODUCTION

A subunit vaccine against the viral haemorrhagic septicaemia virus (VHSV) would be most convenient to slow down one of the most damaging diseases in the worldwide salmoniculture industry. Attempts to obtain it, however, have not yet been really successful (Leong et al. 1995, Lorenzen & Olesen 1997). The viral glycoprotein G (gpG) elicits neutralizing antibodies and has also been shown to be an efficient stimulator of leucocyte proliferation *in vitro* (Estepa et al. 1994, Lorenzo et al. 1995). As the gpG appears to activate humoral as well as cellular defense mechanisms, it seems to be an obvious candidate for a recombinant vaccine. However, the recombinant gpG of VHSV conferred no or moderate protection against VHSV by immersion when expressed in bacterial cells such as *Escherichia coli* (Lorenzen & Olesen 1997), *Aeromonas salmoni-*

cida (Noonan et al. 1995) or *Yersinia ruckeri* (Estepa et al. 1994). Furthermore, only moderate protection was obtained by injection of the gpG when expressed in insect cells (Lecocq-Xhonneux et al. 1994) or by immersion on gpG when expressed in yeast (Estepa et al. 1994).

Reduction of the disulfide bonds of the gpG inhibited the binding of many neutralizing monoclonal antibodies (MAbs) to rhabdoviruses such as rabies (Benmansour et al. 1991), vesicular stomatitis virus, VSV (Keil & Wagner 1989), VHSV (Lorenzen et al. 1990, Bearzotti et al. 1995) and infectious haematopoietic necrosis virus, IHNV (Kim et al. 1994, Huang et al. 1996). Sequencing of MAb neutralization resistant (MAR) mutants of rabies (Benmansour et al. 1991, Raux et al. 1995), VSV (Vandepol et al. 1986, Luo et al. 1988), IHNV (Kim et al. 1994) and VHSV (Bearzotti et al. 1995) has suggested that 2 regions being distant in the primary structure of the gpG might be associated in the native conformational stage of the protein. Only a few linear reduction-resistant neutralization epitopes

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have been identified in rabies (VanDeerHeijden et al. 1993, Ni et al. 1995), VSV (LeFrancois & Lyles 1982, Volk et al. 1982, Keil & Wagner 1989, Grigera et al. 1992) and IHNV (Leong et al. 1995) but none yet in VHSV (Lorenzen et al. 1990, Sanz & Coll 1992). The present study focusses on mapping of linear antibody epitopes on the gpG of VHSV. Because neutralizing ability of an antibody to a rhabdovirus (i.e. VHSV) in cell culture does not always correlate with its protection properties *in vivo* (LeFrancois 1984) and nonneutralizing anti-VHSV antibodies can be VHS protective when injected *in vivo* (Lorenzen et al. 1990), the present work is also an attempt to identify some of the major antigenic sites (neutralizing or not neutralizing) that can be mimicked by synthetic peptides in the gpG of VHSV. Mapping of the linear antibody epitopes in the gpG could either give information concerning the failure of recombinant gpG as a vaccine and/or identify regions of the protein which could be important to include in a future vaccine.

MATERIALS AND METHODS

Synthetic peptides from the gpG sequence of VHSV. A series of 15-mer peptides overlapping 5 amino acids (aa) and spanning the cDNA derived aa sequence of the gpG (Thiry et al. 1991) of VHSV 07.71 were chemically synthesized (Chiron Mimotopes, Victoria, Australia). The peptides were named by the amino terminal position of their middle aa (8th position) in the protein sequence, the first peptide of the gpG being the number 6 due to needs of synthesis (it contained 2 additional aa before the initial methionine, according to the cDNA sequence). Additional peptides, p2 (aa 82-109), p3 (aa 110-121) and p4 (aa 122-151), were obtained from Clontech (Palo Alto, CA, USA).

Viral haemorrhagic septicaemia virus. The VHSV 07.71 isolated in France (LeBerre et al. 1977) from rainbow trout *Oncorhynchus mykiss* (Walbaum) was used for most of the experiments. The VHSV was grown and assayed for infectivity in epithelial papillosum cyprini (EPC) cells as described previously (Basurco et al. 1991). For production of purified VHSV, confluent cultures of EPC cells were grown in flasks of 150 cm^2 (Costar) and inoculated with 10 plaque forming units (PFU) of VHSV 07.71 per cell. The culture was harvested after 5 to 7 d at 14°C and centrifuged at 12 000 × g for 30 min to remove cell debris. VHSV was precipitated with 7% polyethyleneglycol (PEG) 6000 in 2.3% NaCl, pH 7.8. After overnight agitation at 4°C, the VHSV was pelleted at 20 000 × g for 40 min. The pellet was dissolved in 10 mM Tris, 1 mM EDTA and 100 mM NaCl, pH 7.6 (TEN) and frozen until used. VHSV concentrated by PEG was layered on a 15 to 45% sucrose gradient in TEN and spun at 80 000 × g for 270 min in a Beckman ultracentrifuge. The band containing infectivity at 31% sucrose (Basurco et al. 1991) was further purified by ultracentrifugation through a 20% sucrose cushion and kept at 4°C for a few weeks until used.

Isolation of soluble gpG by affinity chromatography. Soluble gpG (gpGs) was isolated by affinity chromatography over Sepharose-concanavalin A from VHSV-infected and VHSV-free cell culture supernatants (Perez et al. 1998). The affinity chromatography buffer was 0.1 M sodium acetate adjusted to pH 7.6 to which salts were added to the following final concentrations: 1 mM MnCl$_2$, 1 mM MgCl$_2$, 1 mM CaCl$_2$ and 1 M NaCl. About 2 l of PEG supernatants in affinity chromatography buffer was chromatographied through a ConA column (1.5 × 1 cm). Concentrated salts were added to the VHSV-free supernatants to obtain the final concentrations mentioned above. The supernatant was passed more than 8 times through the column with the aid of a peristaltic pump at room temperature. After washing the column with 100 ml of affinity chromatography buffer, the bound gpGs was eluted with 10 ml of 1 M glucopyranoside, 1 M mannopyranoside in chromatography buffer. The gpGs preparations contained ~30% of the molecules as trimers and 70% as monomers of ~60 kDa as shown by ultracentrifugation in sucrose gradients and by PAGE and immunoblotting.

Recombinant VHSV gpG. Fragment gpG4 (aa 9-443) was cloned and expressed in the yeast *Saccharomyces cerevisae* DC04 as reported previously (Estepa et al. 1994). The nucleotide sequences encoding aa 17-191, 153-474 and 360-474 were amplified by PCR using the cloned G gene of VHSV isolate 3592B as a template following the principles outlined earlier (Lorenzen et al. 1993). The vector used for expression under the control of a T7 bacteriophage promotor has been described by Christensen et al. (1991). Using the NdeI and HindIII sites downstream of the T7 promotor, the earlier fusion protein construct including parts of the λcII and myosin light chain genes as fusion partner (Lorenzen et al. 1993) was inserted into the expression vector. Amplified G fragments were subsequently cloned into the vector using the BamH I and Hind III sites of the construct. Expression of the fusion proteins was induced by addition of λCE-6 carrying the T7 polymerase gene, to exponentially growing *Escherichia coli* DH1 cells when the OD$_{600}$ was approx. 0.9. Cultivation was continued for another 3 h and the cells were subsequently harvested by centrifugation. Cell pellets were dissolved by boiling in reducing sample buffer for SDS-PAGE. SDS-PAGE and immunoblotting were performed as before (Lorenzen et al. 1990). Purified virus was used a positive control antigen.

Monoclonal antibodies (MAbs). MAbs against VHSV 07.71 were produced by immunization of Balb/c mice with either recombinant VHSV gpG4 expressed in yeast (30 μg per injection) or with heat-killed whole virus (50 μg per injection). The final boost was carried out by intravenous injection of purified soluble gpGs protein (10 μg per injection). Spleen cells from immunized mice were fused with the myeloma cell lines SP2 or P3-X63-Ag.653 myeloma line (Sanz et al. 1993). Screening of the hybridoma supernatants was performed by indirect ELISA on gpG4 coated plates and by flow cytometry (FACS) on VHSV-infected EPC cells. Culture supernatants from the selected clones were concentrated by ammonium sulfate precipitation or purified by protein A-Sepharose (Pharmacia) following methods described before (Sanz et al. 1993). The concentrated samples were applied onto precoated nitrocellulose membrane strips (ImmunoType Kit, Sigma) to determine the MAbs isotypes by following the procedure recommended by the manufacturers of the kit. Other MAbs used were 3F1H10 and IP1H3 (Lorenzen et al. 1990) and I10 kindly donated by Dr De Kinkelin (Mourton et al. 1990). Neutralizing MAb C10 was obtained from Sanofi Diagnostic Pasteur (Marnes-La-Coquette, France). Neutralizing MAb 3F1A2 (Lorenzen et al. unpubl.) was produced as described earlier (Lorenzen et al. 1990). 1H10 is a neutralizing-enhancing MAb that recognized the denatured form of the gpG (Sanz & Coll 1992). The ability of the MAbs to react with a denatured form of VHSV gpG was tested by Western blot analysis. SDS-acrylamide 12% gels were loaded with 1 mg gpG4 in buffer containing mercaptoethanol. The proteins in the gel were transferred to nitrocellulose membranes (BioRad). 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 one of the purified MAbs (1:100 dilution) before incubation with the peroxidase-conjugated rabbit anti-mouse antibody (1:2000). Finally, the nitrocellulose strips were treated with ECL detection reagents (Amersham), dried, and exposed to X-ray AGFA film.

Production of anti-VHSV gpG polyclonal antibodies in mice. To increase the probability of obtaining a high titre of VHSV neutralizing antibodies, different antigenic preparations were used. Each female BALB/c mouse was first injected with 20 μg of purified gpGs or purified 1% formalin/1 h treated gpGs (Bachmann et al. 1993) in Freund's complete adjuvant. Then 4 injections given monthly with the same antigens in Freund's incomplete adjuvant were carried out. Alternatively, 20 μg of purified VHSV was mixed with 20 μg of saponin (Superfos, Vedbaek, Denmark), sonicated and injected per mouse, for 4 mo with monthly injections, and then, to increase the anti-gpG immune response, 20 μg of gpG4 in Freund's was given to each mouse for 3 more months.

To obtain ~40 ml of pooled diluted ascites, 3 immunized mice per antigen were intraperitoneally injected with 0.5 to 2×10^6 viable myeloma X63/Ag8653 cells per mouse. Mice ascites was then obtained by injection of physiological saline a few days later and pooled as described in Coll (1989). Such ascites contained 20 to 80% of the serum antibody concentration. The pooled ascites was clarified by low speed centrifugation and stored at −40°C until use. The pooled ascites was passed throughout a 3×10 cm Sepharose column (Pharmacia) with bound *Escherichia coli* protein extract (~10 mg ml⁻¹) and rabbit serum (~10 mg ml⁻¹) to adsorb background antibodies. The immunoglobulin G (IgG) from the adsorbed pooled ascites was then purified by affinity chromatography over protA-Sepharose columns (Pharmacia, Uppsala, Sweden) adjusted to 1.5 M glycine, 3 M NaCl, pH 8.9. The retained IgG was eluted with 50 mM ethylenediamine pH 11 and immediately neutralized with 3 M Tris, pH 3.5. Eluted fractions were pooled and dialyzed against 10 mM sodium phosphate, 150 mM NaCl, pH 7.2 (PBS). Purity as tested by gel electrophoresis gave 2 single Coomassie blue stained bands at 50 and 24 kDa, respectively.

Production of anti-VHSV polyclonal antibodies in trout. Trout (T1) of 500 g were injected intraperitoneally 3 times with 30 μg of sucrose-gradient purified VHSV, reference strain DK-F1 passed 254 times in cell culture until loss of pathogenicity. The fish were bled 1 mo after the last injection and the sera were freeze dried in 0.4 ml aliquots (kindly provided by N. J. Olesen). Trout (T3) were also injected 4 times over 3 mo with 30 μg of heat-killed (37°C, 30 min) PEG-concentrated VHSV 07-71 mixed 1:1 with complete Freund's adjuvant for the first injection and mixed 1:1 with incomplete Freund's for the remaining injections (200 μl of total volume per trout per injection).

To generate survivors of VHSV infections, about 400 trout weighing between 0.5 and 2 g were infected by bath for 2 h at 12 to 14°C with 10^6 TCID$_{50}$ ml⁻¹ of VHSV 07.71 attenuated by 10 passages on EPC cells (Basurco & Coll 1992). After 1 mo, survival was between 10 and 30% (4 experiments). The trout surviving the infection were challenged 1 to 3 mo later with low passage VHSV 07.71 isolated on EPC cells from infected trout (10^6 TCID$_{50}$ ml⁻¹, for 2 h at 10 to 11°C). After 1 mo, between 50 and 80% (4 experiments) of the trout had survived this second infection (De Kinkelin 1988) and had no signs of haemorrhagic septicaemia. At 2 to 4 mo after the last VHSV challenge, when the fish weighed 100 to 200 g each and when the frequency of trout with detectable anti-VHSV antibodies has been described to be the highest (Enzmann & Konrad 1993), the fish were bled and serum samples kept at −20°C (T3).

As control sera 3 pools were obtained, from 17 healthy noninfected trout of 20 to 500 g body weight from different farms (T4), from 4 healthy noninfected trout of 20 g body weight maintained in the laboratory (T5) and from a large batch of pooled serum from about 200 trout of 100 to 200 g body weight obtained from a farm with no previous history of VHSV infections (Vistahermosa, León, Spain) (T6). Pools were used to minimize the effect of a few possible positive fish.

Indirect ELISA. Polystyrene plates (Dynatech, Plochingen, Germany) were coated with 300 to 1000 pmol peptides well^{-1} or 1 µg purified VHSV well^{-1} in 100 µl of distilled water, incubated overnight at 37°C to dryness, and kept sealed with blue silica gel at 4°C. The PAbs and MAbs were diluted 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 l^{-1} bovine serum albumin, 0.3% rabbit serum, 0.5 g l^{-1} Tween 20, 50 mg l^{-1} phenol red, pH 6.8) and incubated overnight at 4°C. Preliminary experiments showed that both inclusion of rabbit serum in the dilution buffers and in the blocking step and overnight incubation of the diluted Abs with the dilution buffers reduced nonspecific reactivities. The plates were blocked by incubation for 15 min with dilution buffer. After blocking, the plates were incubated for 60 min at room temperature with 100 µl well^{-1} of the diluted PAbs or MAbs and washed once with distilled water. To detect trout PAbs, the MAb anti-trout IgM 1G7 (Sanchez et al. 1991) was incubated for 30 min at room temperature and washed once with distilled water. Peroxidase-labelled rabbit anti-mouse IgG (Nordic, Tilburg, The Netherlands) 100-fold diluted with dilution buffer was added (100 µl well^{-1}), incubated for 30 min and then washed 3 times with distilled water. For color development, 50 µl of substrate buffer (150 mM sodium citrate, 3 mM H_2O_2 and 1 mg l^{-1} o-phenylenediamine, pH 4.8) was pipetted per well and the reaction stopped with 50 µl well^{-1} of 4 M H_2SO_4 after 30 min. The results were read in a Titertek Multiskan RC at wavelengths of 492 and 620 nm. The absorbance at 620 nm was used to correct for individual nonsignificant differences between wells.

Neutralization assays. About 10^3 TCID$_{50}$ ml^{-1} of VHSV 07.71 was incubated overnight at 4°C with serial dilutions of anti-VHSV antibodies (Lorenzo et al. 1996). Then 100 µl of the different virus/antibody mixtures was added to cultures of EPC cells in 96-well plates, adsorbed for 2 h at 14°C, washed with cell culture medium (RPMI containing 2% of FCS), filled with 100 µl well^{-1} of cell culture medium and incubated overnight at 14°C. The VHSV-infected EPC monolayers were fixed for 10 min in cold methanol and dried. To detect the N antigen of VHSV, the MAb 2C9 1000-fold diluted in ELISA dilution buffer was added

to the wells (100 µl well^{-1}) and incubated for 1 h. After washing by immersion in distilled water, 100 µl of peroxidase-labelled anti-mouse IgG (Nordic) was added per well and incubation continued for 30 min. After 3 washings by immersion in distilled water, 50 µl of 1 mg ml^{-1} of diaminobenzidine (DAB) (Sigma) in the appropriate buffer was added per well, and the reaction was allowed to proceed until brown foci were detected by inspection with an inverted microscope in the controls containing no antibodies. Once washed with water and air dried, brown foci (DAB positive foci) were counted with an inverted microscope.

Flow cytometry. EPC cultures containing 1×10^6 cells 5ml^{-1} flask^{-1} were infected at 10 VHSV-07.71 TCID$_{50}$ cell^{-1}. Noninfected controls were included in every experiment. Incubation was at 14°C for 24 h. EPC cells were detached from the surface of the 25 cm^2 bottles by mechanical agitation in PBS (0.15 M sodium chloride, 0.01 M sodium phosphate, pH 7.4) containing 1% BSA, 5 mM EDTA and 0.1% sodium azide. EPC suspensions were centrifuged at $1200 \times g$ for 10 min and the pellet was gently resuspended in PBS + BSA + azide containing 50-fold diluted mouse ascites with anti-VHSV antibodies or protein A-purified MAbs adjusted to 1 mg ml^{-1}. After 1 h at 20°C with occasional agitation, the cell suspensions were centrifuged again, washed twice, resuspended in 400-fold diluted rabbit anti-mouse IgG-FITC conjugate (Nordic) and incubated for 30 min at 20°C. The EPC suspensions were again centrifuged and washed twice and then resuspended in PBS + 0.3% paraformaldehyde. The same day of the harvest and staining, 5000 cells were analysed by flow cytometry in a Beckton-Dickinson (San José, CA) FACScan apparatus using the program LYSYS II version 1.0. Fluorescence was measured at FL1 (514 to 545 nm, green).

RESULTS

Pepscan mapping with trout sera

Pooled serum from survivors of VHSV and trout injected with purified VHSV (Fig. 1, T3) and individual serum from trout with high VHSV neutralization titres (Fig. 1, T1 and T2) recognized peptide p106. Furthermore T2 strongly bound p206. Increased reactivity was also seen with other peptides but the levels were comparable to those obtained with pooled sera from trout that had no known contact with VHSV (Fig. 1, T4, T5 and T6). The higher reactivity with p106 appeared to be the major difference between the sera pools from trout previously exposed to VHSV and the sera from trout with no previous contact with VHSV.
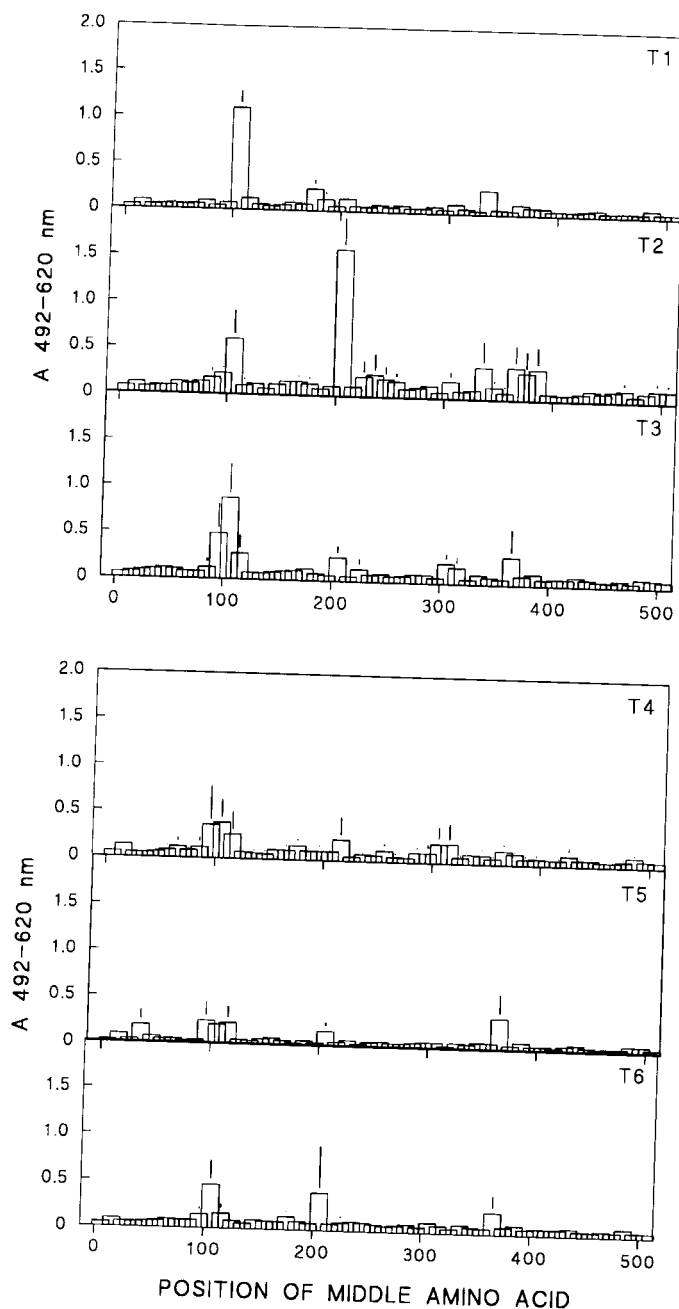
Fig. 1. ELISA reactivity of gpG pepscan with trout serum. Serum from different trout were obtained, pooled, heat inactivated (45°C for 30 min) and centrifuged at $5000 \times g$ for 10 min to eliminate precipitates. Sera supernatants were diluted 1/200 in dilution buffer and ELISA was performed as described. No reaction with the peptides was obtained if the step with the MAb 1G7 was omitted. T1: serum from a trout injected with sucrose-gradient-purified VHSV with a neutralization titre of 300 000 (provided by Dr Olesen); T2: serum from a trout surviving VHSV with a neutralization titre of 100 000 (provided by Dr De Kinkelin); T3: pooled serum from 6 trout survivors of VHSV infection plus 3 trout injected with PEG-purified VHSV; T4: pooled serum from 17 noninfected healthy trout from different farms; T5: pooled serum from 4 noninfected healthy trout maintained in the laboratory; T6: pooled serum from ~200 noninfected healthy trout from 1 farm

Pepscan mapping with mice anti-VHSV PAbs

Mice were hyperimmunized with purified soluble gpG (gpGs), either fixed with formaldehyde (fgpGs) or native (ngpGs), and with purified VHSV plus recombinant gpG4. Preliminary ELISA experiments showed that the reactivity of the pepscan peptides with the anti-VHSV murine ascites was very low. To obtain measurable absorbance values, IgG had to be isolated and concentrated from the ascites. Relative ELISA titres (measured against pgG4) of anti-VHSV IgG were higher when fgpGs or ngpGs had been used for immunization compared to when VHSV+ gpG4 had been used. However, neutralization titres were higher for ascites obtained by using VHSV+ gpG4 than for ascites obtained by using gpGs as antigen (Table 1).

When the anti-VHSV IgG obtained when using ngpGs and VHSV+gpG4 were tested against the gpG pepscan, the most antigenic peptides were p96, p106, the region from p176 to p236, p306 and the region from p366 to p386 (Fig. 2). Most of these regions could also be detected by using fixed gpGs (Fig. 2, fGs) but with more difficulties since the overall reaction with most of the peptides seems to be unspecifically increased. The synthetic peptides covering the signal sequence (aa 6–26) and the transmembrane region (aa 462–482) absent from both gpG antigens (gpGs and gpG4) show the estimated average level of background (<0.2). The results obtained with MAb 1G7 (anti-trout IgM) obtained and processed in the same way as the other PAbs did not show any absorbance value ≥ 0.15 for any of the peptides of the pepscan.

Pepscan mapping with anti-gpG MAbs

The antigenicity of the pepscan peptides was further studied with anti-gpG MAbs (Table 2) by ELISA. I10 reacted with p146 whereas IP1H3 reacted with p406 (Fig. 3). The results obtained with the pepscan for MAbs I10 and IP1H3 were confirmed by Western blot using truncated recombinant gpG fusion proteins. MAb I10 bound the gpG fragment including aa 17–191, whereas MAb IP1H3 recognized fragments including aa 360–474 and aa 153–474 (Fig. 4). The results obtained with MAb I10 were further confirmed by ELISA using other synthetic peptides. Thus whereas I10 showed positive reaction with p4 (aa 122–151), it did not show any reaction with p2 (aa 82–109) or with p3 (aa 110–121) (not shown). Other MAbs (Table 2) did not show any reactivity in the pepscan (not shown).

Table 1. Relative titres of anti-VHSV gpG murine PAbs. IgG from mice ascites was isolated by prot-A affinity chromatography and concentrated to 1 mg ml^{-1} of protein. ELISA titre was then tested against purified VHSV and defined as the absorbance at 492 nm ($A_{492\ nm}$) per µg of protein as calculated from the linear part of the absorbance/dilution curve. Neutralization titre was defined as the inverse of the concentration of IgG necessary to reduce to 50% the number of VHSV-infected foci. Averages from duplicates are shown. gpGs: glycoprotein G isolated from the supernatant of VHSV-infected EPC (s: soluble). ngpGs: native gpGs. fgpGs: formaldehyde-fixed gpGs

Antigen	Adjuvant	ELISA titre ($A_{492\ nm}$ µg^{-1} protein)	Neutralization titre (1/µg ml^{-1})
VHSV+gpG4	Saponine	0.11 ± 0.03	0.51 ± 0.32
ngpGs	Freund's	0.40 ± 0.05	0.014 ± 0.001
fgpGs	Freund's	0.36 ± 0.12	0.012 ± 0.001
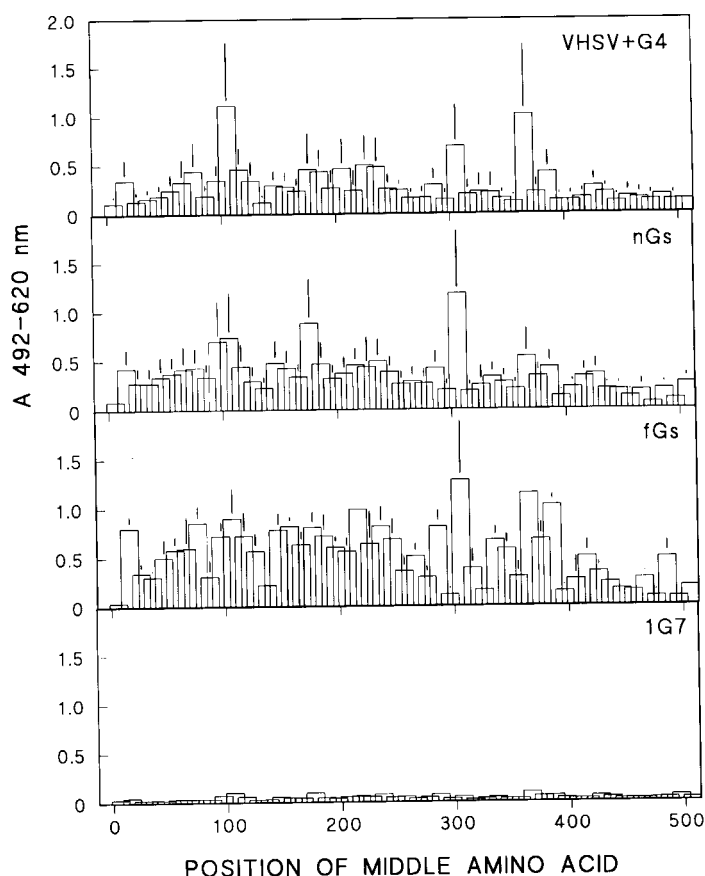
Fig. 2. ELISA reactivity of gpG pepscan with mice anti-G PAbs. 15-mer peptides from the gpG of VHSV in solid phase were incubated with 15 µg ml^{-1} (except fGs which was 5 µg ml^{-1}) of purified IgG obtained from mice ascites containing anti-gpG PAbs. fGs: ascites from mice immunized with fixed Gs with Freund's adjuvant; nGs: ascites from mice immunized with native Gs with Freund's adjuvant; VHSV + G4: ascites from mice immunized with purified VHSV and G4 with saponine adjuvant; 1G7 IgG: isolated from mice ascites containing anti-trout IgM MAb 1G7 (Sanchez et al. 1991) used as a control. Means and standard deviations from 3 different experiments are represented

Flow cytometry of VHSV-infected cells with anti-gpG MAbs

Flow cytometry with VHSV-infected EPC cells showed that the 2 non-neutralizing pepscan-positive MAbs I10 and IP1H3 were unable to stain the surface of infected cells, suggesting that the epitopes they recognize are not available when the gpG is native and situated on the surface of the infected cells. In contrast, neutralizing pepscan-negative MAbs 1H10, C10 and 3F1A2 were capable of staining the surface of VHSV-infected EPC cells, indicating that the epitopes recognized by these MAbs are exposed on the surface of the membranes of VHSV-infected cells (Fig. 5).

DISCUSSION

The mapping of linear epitopes recognized by anti-VHSV PAbs obtained from trout and/or mice indicates

Table 2. Properties of the MAbs against the gpG of VHSV in this work. *MAbs obtained with the myeloma SP2. The rest were obtained with the myeloma line X63-Ag8.653. MAbs were obtained in ascites and protein A purified as described in the 'Materials and methods'. ELISA titre was defined as the inverse of the dilution of the MAb that yields an OD$_{(492\ nm)}$ more than 2-fold over the background. % neutralization = 100 − (number of foci with the MAb − background)/(number foci in control − background) × 100. (+) Positive result, (−) negative result. nd: not determined. To obtain the % of neutralization shown in the table, MAbs C10 and 3F1A2 were used at 1 µg ml^{-1}, MAb 1H10 was used at 500 µg ml^{-1}, MAb 1C3 was used at 65 µg ml^{-1} and MAb 4E6 was used at 10 µg ml^{-1}. The following MAbs had been described previously: I10 (Mourton et al. 1990), IP1H3 (Lorenzen et al. 1990), 1H10 (Sanz & Coll 1992), and C10 (Mourton et al. 1990)

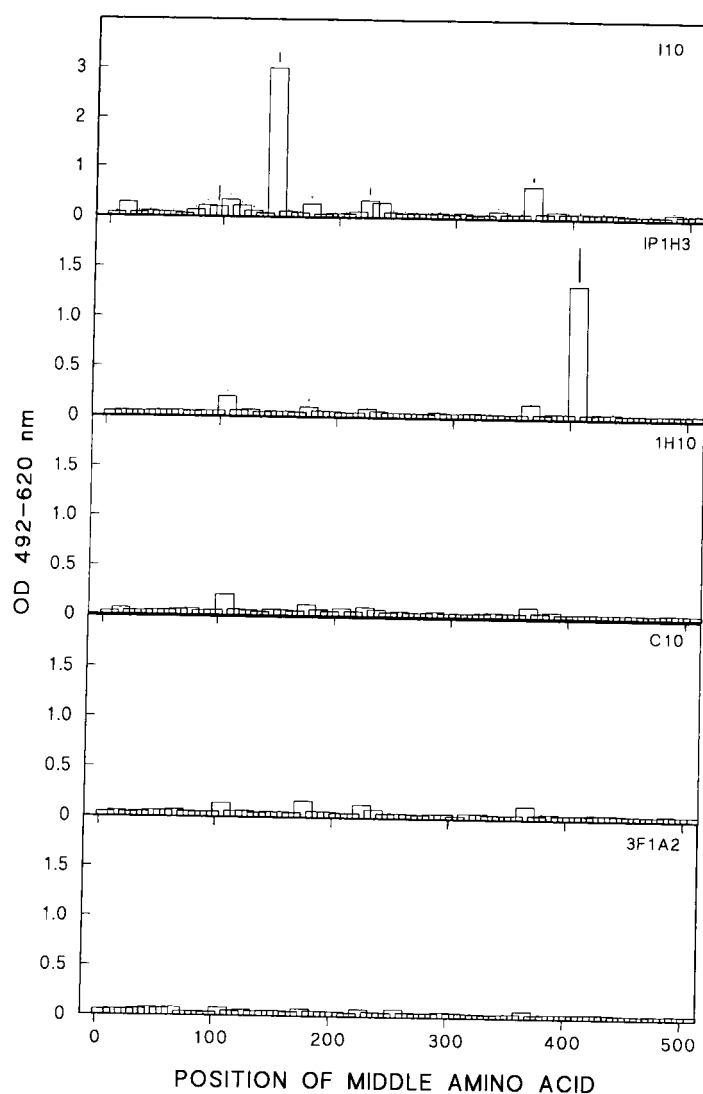| Name	(Isotype)	ELISA G4	ELISA VHSV	Blot G4	FACS	% neutralization
I10	(−)	>1000	>1000	+	nd	−
IP1H3	(IgG1)	>1000	>1000	+	nd	−
1H10	(nd)	>1000	>1000	+	+	80
C10	(IgG2a)	20	120	−	+	100
3FIA2	(IgG1)	20	120	−	+	100
1C3	(IgG1)	500	2500	−	−	77
1G2	(nd)	100	20	+	−	−
1G5	(IgG2a)	100	20	−	−	−
2D1	(IgG2a)	100	−	+	−	−
2G4*	(nd)	20	−	−	−	−
3B8*	(nd)	20	−	−	−	−
3C12*	(nd)	20	−	−	−	−
3F4	(nd)	20	−	−	−	−
4B10*	(IgG3)	500	2500	−	−	−
4E5*	(IgG2a)	20	−	−	−	−
4E6*	(IgM)	20	−	−	+	36
4E7*	(nd)	100	−	−	−	−

Fig. 3. ELISA reactivity of gpG pepscan with anti-gpG MAbs. 15-mer peptides from the gpG of VHSV in solid phase were incubated with purified IgG isolated from murine ascites containing anti-gpG MAbs. Means and standard deviations from 2 different experiments are shown. MAbs were I10 and C10 (Mourton et al. 1990), IP1H3 (Lorenzen et al. 1990), 1H10 (Sanz & Coll 1992, Sanz et al. 1993) and 3F1A2 (Lorenzen unpubl.). MAbs 1H10, C10 and 3F1A2 were neutralizing. Similar results to those obtained with MAbs 1H10, C10 and 3FIA2 were obtained with other MAbs (Table 2)

Fig. 4. Immunoblotting reactivity of MAbs with truncated VHSV gpG expressed in *E. coli*. G (67 kD), N (41kD), M_1 (28 kD), M_2 (24 kD), VHSV proteins on the left-hand side. Lanes 1: purified VHSV; lanes 2 to 4: *E. coli* cell extract containing fusion proteins including aa 17–191 (lanes 2), 153–474 (lanes 3) and 360–474 (lanes 4) of the gpG. A: stained with colloidal gold for total protein. B: immunostained with MAb IP1H3. C: immunostained with MAb I10

the presence of at least 2 regions in the gpG of VHSV which map outside the mutant aa positions identified by the sequencing of MAR mutants (Bearzotti et al. 1995). The region situated around p106 appears antigenic not only using mice PAbs but also with trout PAbs (Figs. 1 & 2). This region contained the amino heptad repeat region which was the highest phosphatidylserine binding region of VHSV (Estepa & Coll 1996) and including some trout leucocyte stimul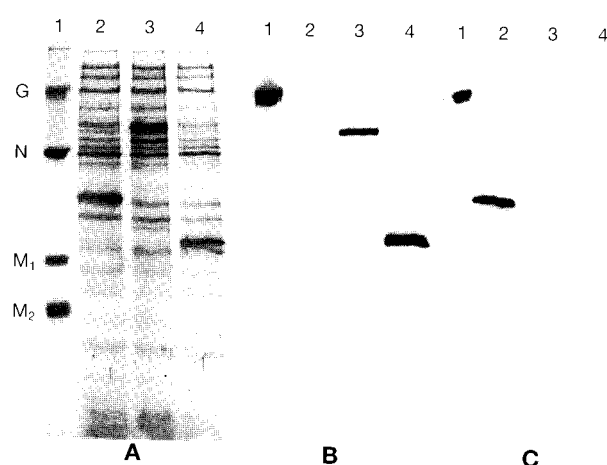ating sequences (Lorenzo et al. 1995). The other antigenic region situated between aa 300 and 400 contained the other 2 heptad repeats of VHSV, some lower phosphatidylserine binding stretches (Estepa & Coll 1996) and another leucocyte stimulating peptide (p306) present in 41.6% of an outbred trout population (Lorenzo et al. 1995). Nevertheless, by performing the pepscan assays in solid phase rather than in solution because of practical feasibility, we have probably bypassed some possible reactivities. All of this study is thus restricted by the methodology used and no claims of exclusivity can be made.

It is not surprising that serum from trout that have no previous contact with VHSV gave some positive reactions in the pepscan (Fig. 1, T4, T5 and T6) because it is well known that trout serum tend to bind nonspecifically to many surfaces and/or proteins (Olesen et al. 1991, Sanchez et al. 1993). On the other hand, the possibility cannot be excluded that a few trout included in those uninfected control trout pools might have survived a previous unnoticed VHS disease. Further studies including higher numbers of immunized and non-immunized fish are needed to analyze this variability.

Of all the 17 anti-VHSV G MAbs tested in this work, only MAbs I10 (aa 139–153) and IP1H3 (aa 399–413) bound peptides on the pepscan and were positive in immunoblotting. The immunoblot data thus supported the more precise epitope localizations obtained by pepscan but it cannot be taken as indicative of a positive reaction in the pepscan ELISA since MAbs 1G2 and 2D1 also recognize gpG4 in blotting yet they were still negative in the pepscan ELISA, most probably due to their low titre (Table 2).
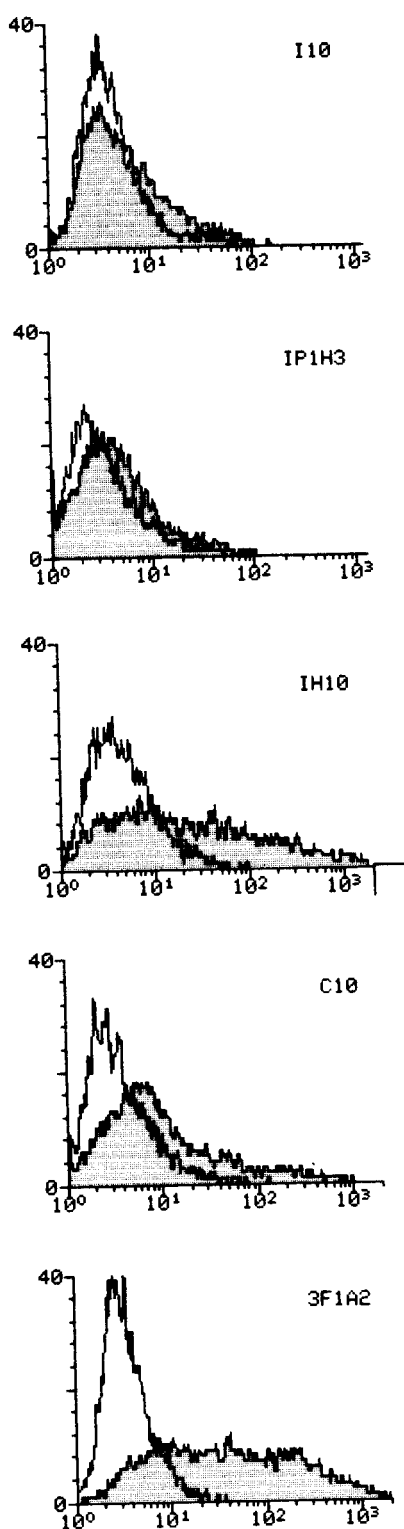
Fig. 5. Fluorescence at 515 to 545 nm measured by flow cytometry of VHSV-infected EPC cells stained with MAbs anti-VHSV I10, IP1H3, 1H10, C10 and 3F1A2. Fluorescence intensity (*x*-axis) versus number of cells (*y*-axis). *x* and *y* axes are in relative units. Shadowed profile: VHSV-infected EPC cells; open profile: noninfected EPC cells

MAbs I10 and IP1H3 did not react by FACS with VHSV-infected cells. However, there was no VHSV-neutralizing activity associated with these MAbs. This result could mean that I10 and IP1H3 were unable to bind to the native gpG, indicating that such epitopes were only accessible to the denatured protein and that, in the membrane of the infected cells, these sites of the gpG could thus be buried (as suggested by the flow cytometry results). In contrast, neutralizing MAbs do bind to the gpG exposed in the membranes of VHSV-infected cells (immunofluorescence positives) but do not bind to the peptides in the pepscan.

The epitopes recognized by I10 and IP1H3 are probably of lineal nature, in contrast to the epitopes recognized by the other gpG MAbs found negative in the pepscan. MAb C10 MAR mutants showed the simultaneous occurrence of at least 2 mutations (most of them at aa 140 and 433) that also appeared in attenuated variants (Bearzotti et al. 1995). It is noteworthy that both of these 2 positions map outside the VHSV epitopes defined in this work by pepscan. However, this result also suggests the conformational nature of that domain. Should these observations be generalized as more results are provided or more MAbs become available, it would suggest the complementarity of both strategies to define antibody epitopes in the gpG of VHSV as has been shown in rabies (Lafay et al. 1996). Complex discontinuous antigenic epitopes involved in VHSV neutralization could be mapped by the MAR mutant strategy whereas linear epitopes could be mapped with the pepscan or similar strategies. Because antigenic regions other than those involved in neutralization *in vitro* could be of importance during *in vivo* protection, as demonstrated for rabies (LeFrancois 1984) and for VHSV (Lorenzen et al. 1990), the identification of linear epitopes could also be of importance for the design of subunit vaccines.

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