Journal of Fish Diseases 1999, 22, 219–222

Short communication

G disulphide bond native conformation is required to elicit trout neutralizing antibodies against VHSV

M Fernandez-Alonso[1], F Alvarez[1], A Estepa[2] and J M Coll[1]

1 INIA, Sanidad Animal CISA, Valdeolmos, Madrid, Spain
2 Centro de Biologia Molecular y Celular, Universidad Miguel Hernandez, Elche, Alicante, Spain

An efficient vaccine against viral haemorrhagic septicaemia (VHSV), a devastating disease of farmed salmonids caused by a virus, would be useful in its control. However, attempts to obtain a recombinant subunit vaccine against VHSV have not yet been completely successful (Leong, Bootland, Anderson, Chiou, Drolet, Kim, Lorz, Mourich, Ormonde, Perez & Trobridge 1995). To that end, the gene coding for the viral glycoprotein G (gpG), the VHSV protein which elicits trout neutralizing antibodies (Lorenzen, Olesen & Vestergaard-Jorgensen 1990) and the major inducer of trout T-cell-like immunoproliferation (Estepa, Thiry & Coll 1994; Lorenzo, Estepa, Chilmonczyk & Coll 1995) has been cloned, sequenced (Thiry, Lecoq-Xhonneux, Dheur, Renard & de Kinkelin 1991; Lorenzen, Olesen, Vestergaard-Jorgensen, Etzerodt, Holtet & Thorgersen 1993b), expressed in different recombinant systems and assayed in trout for induced protection against VHSV. However, the recombinant gpG of VHSV conferred no or moderate protection in trout against VHSV when expressed in bacterial cells such as *Escherichia coli* (Lorenzen *et al.* 1993b), *Aeromonas salmonicida* (Noonan, Enzmann & Trust 1995) or *Yersinia ruckeri* (Estepa *et al.* 1994). Furthermore, only moderate protection in trout against VHSV was obtained by injection of the gpG when expressed in insect cells (Lecocq-Xhonneux, Thiry, Dheur, Rossius, Vanderheijden, Martial & de Kinkelin 1994) or by immersion in gpG4 when expressed in yeast (Estepa *et al.* 1994).

The capacity of VHSV gpG to bind antibodies (antigenic properties) has been well studied. Thus, murine neutralizing antibodies reactive with the gpG recognize conformational disulphide bond-dependent epitope(s) (Lorenzen *et al.* 1990). Also, neutralizing serum from trout injected with gpG, or with inactivated virus particles and survivors of VHSV infection did not recognize gpG after reduction of its disulphide bonds (Lorenzen, Olesen & Jorgensen 1988; Lorenzen *et al.* 1990; Olesen, Lorenzen & Jorgensen 1993). Furthermore, MAb C10-resistant (MAR) mutants showed the simultaneous occurrence of at least two mutations situated well apart in the gpG sequence (aa 140 and 433), mutations that also appeared in attenuated VHSV variants (Bearzotti, Monnier, Vende, Grosclaude, de Kinkelin & Benmansour 1995), suggesting the requirement for a conformation-dependent epitope for neutralization by murine antibodies. Thus, a disulphide intact conformation appears to be a requirement for the antigenic properties of this protein. That an intact disulphide conformation of the gpG is also needed to induce trout neutralizing antibodies (correct immunogenicity) is suggested by the limited success of the immunization attempts against VHSV with the recombinant products mentioned above. However, only a few studies have addressed this point indirectly (Basurco, Yun & Hedrick 1993; Lorenzen, Olesen & Jorgensen

Correspondence *J M Coll, INIA, Sanidad Animal CISA, Valdeolmos 28130, Madrid, Spain. E-mail: coll@inia.es*

1993a; LaPatra, Lauda, Jones, Walker & Shewmaker 1994; Emmenegger, Landolt, LaPatra & Winton 1997).

To study the immunogenicity of gpG with disrupted or preserved disulphide bonds, the present authors used gpG4 inclusion bodies made in yeast which have intermolecular disulphide bonds (Estepa *et al.* 1994; Estepa & Coll 1996) and gpG on purified VHSV prepared with either oil or saponin adjuvants, both of which preserve the disulphide bond structure. The present study demonstrates that, whereas a gpG with intact disulphide bonds is necessary to obtain both binding and neutralizing trout antibodies, immunization with gpG4 induced only trout binding antibodies.

The VHSV-07.71 isolated in France (LeBerre, De Kinkelin & Metzger 1977) from rainbow trout, *Onchorynchus mykiss* (Walbaum), was grown and assayed for infectivity in epithelioma papullosum cyprini (EPC) cells, and PEG-concentrated, as described previously (Basurco, Sanz, Marcotegui & Coll 1991). Recombinant gpG4 (aa 9–443) and N3 were cloned and expressed in the yeast *Saccharomyces cerevisae* DC04 and partially purified, as reported previously (Estepa *et al.* 1994).

Rainbow trout (body weight = 200–500 g) were held in 100-L aquaria at 12–18 °C. Two trout were injected intraperitoneally four times over 3 months with 400 mL of each of the gpG4/VHSV antigen preparations. It was not feasible to prepare sufficient recombinant proteins to immunize more than two trout per preparation. Thirty milligrams of heat-killed (37 °C, 30 min), PEG-concentrated VHSV or of recombinant gpG4 + N3 were diluted 1:1 with complete Freund's adjuvant during the first injection and the rest of the injections were diluted 1:1 with incomplete Freund's. The same preparation of VHSV (30 mg mL^{-1}) was sonicated in the presence of 30 mg mL^{-1} of saponin (Superfos, Quil A), 620 mg mL^{-1} of cholesterol and 6 mg mL^{-1} of phosphatidylcholine (Morein, Sundquist, Hoglund, Dalsgaard & Osterhaus 1984; Larsson, Lovgren & Morein 1993).

To assay for trout binding anti-gpG antibodies by ELISA, polystyrene plates (Dynatech, Plochingen, Germany) were coated with 1 mg of gpG4 per well in 100 mL of distilled water at 37 °C overnight. The plates were blocked just prior to use by incubation for 15 min with dilution buffer. The trout antibodies were diluted 20–2500-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 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). The plates were incubated for 60 min at room temperature with 100 mL $well^{-1}$ of trout antibodies and washed once with distilled water. The material was then incubated for 30 min with antitrout immunoglobulin monoclonal antibody 1G7 (Sanchez, Coll & Dominguez 1991), washed and incubated for 30 min with peroxidase-labelled rabbit antimouse IgG (Nordic, Tilburg, The Netherlands), 100-fold diluted with dilution buffer (100 mL $well^{-1}$) and then washed three times with distilled water. For colour development, 50 mL of 150 mM sodium citrate, 3 mM H_2O_2 and 1 mg L^{-1} o-phenylenediamine, pH 4.8, were pipetted per well, and the reaction was 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 (Sanz & Coll 1992).

To assay for trout anti-VHSV neutralizing antibodies, 10^3 $TCID_{50}$ mL^{-1} of VHSV 07.71 were incubated overnight at 4 °C with serial dilutions of trout serum (Lorenzo, Estepa & Coll 1996). Then 100 mL of the different virus/antibody mixtures were added to cultures of EPC cells in 96-well plates, adsorbed for 2 h at 14 °C with agitation, washed with cell culture medium (RPMI containing 2% foetal calf serum), refilled with 100 mL $well^{-1}$ 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, MAb 2C9 (Sanz & Coll 1992) 1000-fold diluted in ELISA dilution buffer was added to the wells (100 mL $well^{-1}$) and incubated for 1 h. After washing by immersion in distilled water, 100 mL of peroxidase-labelled antimouse IgG were added per well and incubation continued for 30 min. After washing, 50 mL of 1 mg mL^{-1} per well of diaminobenzidine (DAB) (Sigma Chemical Company, St Louis, MO, USA) in the appropriate buffer was added until brown foci were detected with an inverted microscope in the controls containing no trout antibodies. Once washed with water and air dried, the brown foci (DAB positive foci) were counted with an inverted microscope.

Anti-gpG binding antibodies, estimated by ELISA, showed a two- to three-fold higher titre in the serum obtained from trout immunized with gpG4 + N3 than in the serum obtained from trout

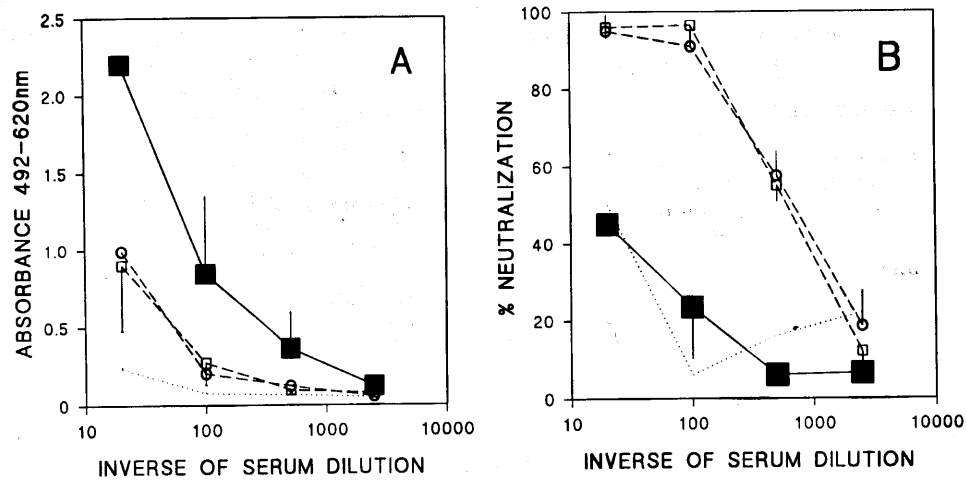

Figure 1 (a) Anti-gpG-binding trout antibodies estimated by ELISA using plates coated with gpG4. (b) VHSV-neutralizing trout antibodies estimated by using a neutralization test. The results from two rainbow trout per immunization were averaged and standard deviations were calculated: (■) serum from trout immunized with gpG4 + N3 proteins; (○) serum from trout immunized with VHSV in Freund's adjuvant; (□) serum from trout immunized with VHSV in saponin; and (....) serum from non-immunized trout or trout injected only with adjuvants.

immunized with VHSV in either oil adjuvant or saponin (Fig. 1a). No neutralizing antibodies could be detected in serum obtained from trout immunized with gpG4 + N3, whereas neutralizing antibodies were obtained in serum from trout immunized with VHSV in either oil adjuvant or saponin (Fig. 1b).

The serum obtained from trout immunized with gpG4 + N3, or VHSV in oil adjuvant, showed the presence of antibodies to both gpG and N VHSV proteins. The serum obtained from trout immunized with VHSV in saponin showed only the presence of antibodies to gpG, as estimated by immunoblotting (data not shown). Because only the gpG has a transmembrane domain, it is the only VHSV protein which could form a complex with the saponin (iscom), and therefore, it is only the VHSV protein which could be immunogenic when this adjuvant is used (Larsson *et al.* 1993).

The trout antibodies which reacted strongly by ELISA with gpG4 have no VHSV-neutralizing activity, which probably means that these were unable to bind to the native gpG protein and that such neutralizing epitopes were not accessible in the disulphide-disrupted protein. Numerous previous studies on the antigenicity of the gpG have shown that neutralizing epitopes of VHSV are disulphide-bond dependent (antigenic properties). On the other hand, the present results also highlight the inability to obtain conformation-dependent neutralizing antibodies by immunizing with a disulphide-disrupted form of the gpG, the gpG4 (immunogenic properties).

In contrast, when the disulphide bonds were preserved during the preparation of the gpG immunogen (either by using oil adjuvant or saponin), neutralizing antibodies could be obtained even though this trout sera contained a lower amount of binding anti-gpG antibodies (Fig. 1). Most probably this lower titre of binding antibodies could be a result of the lower concentration (about 10-fold lower) of gpG in the concentrated VHSV compared to the gpG4 concentration used for immunization. Although these results should be regarded only as preliminary because of the extremely small numbers of fish which could be used, taken together with previous studies, the findings confirm that both the antigenic and the immunogenic neutralizing properties of the gpG require a disulphide intact conformation.

Acknowledgments

Thanks are due to J. P. Coll for assistance with the typing of the manuscript. This work was supported by CICYT projects AGF95-910, AGF97-297 and AGF98-580, Spain, and by FAIR projects CT98-4398 and CT98-4003 from the European Union. M.F. was the recipient of a fellowship from the

Gobierno vasco, Departamento de Educación, Universidades e Investigación, Spain.

References

Basurco B., Sanz F., Marcotegui M.A. & Coll J.M. (1991) The free nucleocapsids of the viral haemorrhagic septicaemia virus contain two antigenically related nucleoproteins. *Archives of Virology* **119,** 153–163.

Basurco B., Yun S. & Hedrick R.P. (1993) Comparison of selected strains of infectious hematopoietic necrosis virus (IHNV) using neutralizing trout antisera. *Diseases of Aquatic Organisms* **15,** 229–233.

Bearzotti M., Monnier A.F., Vende P., Grosclaude J., de Kinkelin P. & Benmansour A. (1995) The glycoprotein of viral hemorrhagic septicemia virus (VHSV): antigenicity and role in virulence. *Veterinary Research* **26,** 413–422.

Emmenegger E., Landolt M., LaPatra S. & Winton J. (1997) Immunogenicity of synthetic peptides representing antigenic determinants on the infectious hematopoietic necrosis virus glycoprotein. *Diseases of Aquatic Organisms* **28,** 175–184.

Estepa A. & Coll J.M. (1996) Pepscan mapping and fusion related properties of the major phosphatidylserine-binding domain of the glycoprotein of viral hemorrhagic septicemia virus, a salmonid rhabdovirus. *Virology* **216,** 60–70.

Estepa A., Thiry M. & Coll J.M. (1994) Recombinant protein fragments from haemorrhagic septicaemia rhabdovirus stimulate trout leucocyte anamnestic *in vitro* responses. *Journal of General Virology* **75,** 1329–1338.

LaPatra S.E., Lauda K.A., Jones G.R., Walker S.C. & Shewmaker W.D. (1994) Development of passive immunotherapy for control of infectious hematopoietic necrosis. *Diseases of Aquatic Organisms* **20,** 1–6.

Larsson M., Lovgren K. & Morein B. (1993) Immunopotentiation of synthetic oligopeptides by chemical conjugation to iscoms. *Journal of Immunological Methods* **162,** 257–260.

LeBerre M., de Kinkelin P. & Metzger A. (1977) Identification sérologique des rhabdovirus des salmonidés. *Bulletin de l'Office International des Epizooties* **87,** 391–393.

Lecocq-Xhonneux F., Thiry M., Dheur I., Rossius M., Vanderheijden N., Martial J. & de Kinkelin P. (1994) A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout. *Journal of General Virology* **75,** 1579–1587.

Leong J.C., Bootland L., Anderson E., Chiou P.W., Drolet B., Kim C., Lorz H., Mourich D., Ormonde P., Perez L. & Trobridge G. (1995) Viral vaccines for aquaculture. *Journal of Marine Biotechnology* **3,** 16–23.

Lorenzen N., Olesen N.J. & Jorgensen P.E.V. (1988) Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. *Diseases of Aquatic Organisms* **4,** 35–42.

Lorenzen N., Olesen N.J. & Jorgensen P.E.V. (1993a) Antibody response in rainbow trout to VHS virus proteins. *Fish and Shellfish Immunology* **3,** 461–473.

Lorenzen N., Olesen N.J. & Vestergaard-Jorgensen P.E. (1990) Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein. *Journal of General Virology* **71,** 561–567.

Lorenzen N., Olesen N.J., Vestergaard-Jorgensen P.E., Etzerodt M., Holtet T.L. & Thorgersen M.C. (1993b) Molecular cloning and expression in *Escherichia coli* of the glycoprotein gene of VHS virus and immunization of rainbow trout with the recombinant protein. *Journal of General Virology* **74,** 623–630.

Lorenzo G.A., Estepa A., Chilmonczyk S. & Coll J.M. (1995) Different peptides from haemorrhagic septicemia rhabdoviral proteins stimulate leucocyte proliferation with individual fish variation. *Virology* **212,** 348–355.

Lorenzo G., Estepa A. & Coll J.M. (1996) Fast neutralization/immunoperoxidase assay for viral haemorrhagic septicemia with anti-nucleoprotein monoclonal antibody. *Journal of Virological Methods* **58,** 1–6.

Morein B., Sundquist B., Hoglund S., Dalsgaard K. & Osterhaus A. (1984) ISCOM, a cagelike immunostimulating complex of membrane proteins. In: *Modern Approaches to Vaccines,* Vol. 1 (ed. by R.M. Chanock & R.A. Lerner), pp. 363–367. Cold Spring Harbour Laboratory, New York, NY.

Noonan B., Enzmann P.J. & Trust T.J. (1995) Recombinant infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus glycoprotein epitopes expressed in *Aeromonas salmonicida* induce protective immunity in rainbow trout (*Onchorynchus mykiss*). *Applied and Environmental Microbiology* **61,** 3586–3591.

Olesen N.J., Lorenzen N. & Jorgensen P.E.V. (1993) Serological differences among isolates of viral haemorrhagic septicemia virus detected by neutralizing monoclonal and polyclonal antibodies. *Diseases of Aquatic Organisms* **16,** 163–170.

Sanchez C., Coll J.M. & Dominguez J. (1991) One step purification of rainbow trout immunoglobulin. *Veterinary Immunology and Immunopathology* **27,** 383–392.

Sanz F.A. & Coll J.M. (1992) Detection of hemorrhagic virus of salmonid fishes by use of an enzyme-linked immunosorbent assay containing high sodium chloride concentration and two concompetitive monoclonal antibodies against early viral nucleoproteins. *American Journal of Veterinary Research* **53,** 897–903.

Thiry M., Lecoq-Xhonneux F., Dheur I., Renard A. & de Kinkelin P. (1991) Sequence of a cDNA carrying the glycoprotein gene and part of the matrix protein M2 gene of viral haemorrhagic septicaemia virus, a fish rhabdovirus. *Biochimica et Biophysica Acta* **1090,** 345–347.