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Monoclonal antibodies against the structural proteins of viral haemorrhagic septicaemia virus isolates

F. SANZ, B. BASURCO, M. BABIN, J. DOMINGUEZ & J. M. COLL *Departmento de Sanidad Animal, Centro de Investigación y Tecnología del I.N.I.A., Madrid, Spain*

Abstract. Five VHSV isolates from different host species and Spanish geographical locations and three viral haemorrhagic septicaemia virus (VHSV) international reference serotypes (F_1, F_2 and 23·75) were studied by several characterized monoclonal antibodies (MAbs) including a neutralizing MAb to four structural proteins of VHSV. We report here the lack of reaction between anti-M1 and some of the isolates of VHSV and the homogeneity of most of the isolates with respect to the MAbs tested. The reagents obtained will improve diagnostic tests which currently use polyclonal antibodies.

Introduction

Viral haemorrhagic septicaemia is a viral disease of salmonid fishes causing severe damage in fish farms in Europe (de Kinkelin 1972). The viral haemorrhagic septicaemia virus (VHSV) is a membrane-enclosed, negative strand RNA virus which buds from the infected cell membrane.

The five virion proteins of VHSV which have been identified (Deuter & Enzmann 1986) are designated by the letters, L (the RNA-dependent RNA polymerase of 150–200 kDa), N (the majority phosphorylated nucleoprotein of 45–50 kDa), M_1 and M_2 (the matrix proteins of 22–28 kDa) and G (the neutralizing epitope-carrier glycoprotein of 60–80 kDa).

The existence of a least two serotypes (F_1 and F_2) of VHSV was demonstrated by using antisera in neutralization tests with 76 virus isolates (Vestergard-Jorgensen 1972), Rare new serotypes, designed 23·75 or F_3 and F_4 were also described (Mourton, Bearzotti, Piechaczyk, Paulucci, Pau, Bastide & de Kinkelin 1990). VHSV serotypes seem to have no antigenic relationships with infectious haematopoietic necrosis virus (IHNV) (Mc Allister, Fryer & Pilcher 1974).

Previous attempts to produce monoclonal antibodies (MAbs), either against the proteins of VHSV (Lorenzen, Olesen & Jorgensen 1988) or with neutralizing activity (Lorenzen, Olesen & Jorgensen 1990; Winton, Arakawa, Lannan & Fryer 1988), have stressed the difficulty of obtaining such reagents against L or G proteins. The use of MAbs has recently allowed the recognition of antigenic variants among isolates of IHNV (Winton *et al.* 1988), but similar studies have not yet been reported for VHSV (Lorenzen *et al.* 1988, 1990).

In the experiments reported here, we use a panel of MAbs (including one neutralizing MAb) to study the variability among different VHSV Spanish isolates and international reference strains.

Materials and methods

Viruses

The strains of virus used were VHSV-F_1 and VHSV-F_2, provided by Dr P. E. Vestergard-Jorgensen, VHSV-23·75 provided by Dr P. de Kinkelin and the IHNV-Cedar strain provided

Correspondence: Dr J. M. Coll, Departmento de Sanidad Animal, Centro de Investigación y Tecnología del I.N.I.A., Embajadores 68, 28012-Madrid, Spain.

by Dr R. Hedrick. Five VHSV isolates were obtained from fish tissue samples from Spain (Basurco & Coll 1989a, b). The virus were isolated from: rainbow trout, *Oncorhynchus mykiss* (Walbaum), (689 from Galicia in 1984, 471 from Navarra in 1986 and 144 from Salamanca in 1984); Atlantic salmon, *Salmo salar* L., (472 from Cantabria in 1986); and barbel, *Barbus graellsi* Steindachner, (798 from Aragón in 1986). Unless otherwise indicated, isolate 144 was used throughout the experiments.

Cells, media and virus purification

Epithelioma papillosum cyprini (EPC) cell culture techniques (RPMI-1640 medium, Flow, Ayrshire, Scotland), virus purification and estimation of protein were essentially as reported by de Kinkelin (1972) and modified by Basurco & Coll (1989b). Virus purity, as calculated from scans of Coomassie-blue-stained electrophoresed proteins was about 80% for polyethyleneglycol (PEG)-concentrated virus and 95% for ultracentrifuged virus (Basurco & Coll 1989b). Because the Nx is also part of the viral preparation, it was included in the purity calculations of the PEG-concentrated VHSV (Basurco, Sanz, Marcotegui & Coll 1991). The PEG-concentrated virus, used for immunization, immunoblotting and ELISA, contained 10^{10} $TCID_{50}\,ml^{-1}$ and had a protein content of approximately $5\,mg\,ml^{-1}$. The protein bands of VHSV-144 were identified by Coomassie-blue staining of gels of purified virus, [^{35}S]-methionine-labelled induced viral proteins and immunoprecipitation with international reference polyvalent antisera. All these patterns were identified by comparison with reference serotypes as described by Basurco & Coll (1989a, b). The VHSV proteins were purified by preparative gel electrophoresis of purified VHSV as described previously by Estepa, Basurco, Sanz & Coll (1991).

Immunization of mice

Female mice (BALB/c) were given nine intraperitoneal injections of 25 μg of PEG-concentrated VHS viral protein over a period of 9 months. The injections were given by mixing the virus with equal parts of Freund's adjuvant (only the first injection was in complete Freund's). Five days before fusion, the mice were given two intraperitoneal and one intravenous injections of PEG-concentrated and ultracentrifuged virus on separate days. Antibodies against VHSV were detected by indirect ELISA and immunoblotting (Basurco & Coll 1989b). Spleen cells from the immunized mice were fused with the myeloma cell line P3-X63-Ag 8653. Fusion, cloning twice by limiting dilution, cultivation of hybridoma cells and ascites production were performed as described previously (Martinez & Coll 1988; Rueda & Coll 1988).

Indirect ELISA

The assay used for screening the hybridoma supernatants and to assay for purified VHSV recognition by the MAbs, was essentially as described by Martinez & Coll (1988). Briefly, microtitre plates (Dynatech, Plochingen, Germany) were coated to dryness with 0·5 μg of PEG-concentrated VHSV or purified VHSV proteins, washed for 15 min and dried. Plates coated with PEG-concentrated EPC cell extracts were used as controls. Horseradish peroxidase-conjugated rabbit immunoglobulin to mouse immunoglobulins (Nordic, Tilburg, The Netherlands) was used to develop the reaction between the hybridoma supernatants and the virus coated solid-phase. An ascites (Coll 1987) pool from immunized mice was used as a positive control (×1000 dilution). Development with o-phenylenediamine was by using a low-

background citrate buffer, as described previously by Coll (1989). A well was considered positive when its absorbance at 492 nm in the virus coated wells was 0·3 above the absorbance in the well coated with non-infected EPC cells. The isotype of MAbs was determined by ELISA (Biorad kit for isotype mouse MAb determination, Biorad, Richmond, VA, USA).

Immunoblotting

The protein bands were transferred from 10–15% polyacrylamide gradient gels to nitrocellulose membranes, as described by Coll (1988). After blocking with dilution buffer (0·5% bovine albumin, 0·1% Tween-20, 0·01% merthiolate, 50 mg l^{-1} phenol red in 10 mM sodium phosphate, 0·15 M sodium chloride, pH 7·6), the strips were incubated for 1 h with the hybridoma supernatants diluted 1:2 in dilution buffer. To identify the bands, some strips were stained with Amido-black stain and the rest of the gel was stained with Coomassie-blue. The bands were developed with 1 mg ml^{-1} diaminobenzidine in low background citrate buffer (Coll 1989).

Immunofluorescence

Cover-glass cultures of EPC cells were infected with 10^6 $TCID_{50}$ ml^{-1} of VHSV Spanish isolate 144 (Basurco & Coll 1989a). After washing in medium without serum, the cultures were fixed for 10 min in acetone. The fixed cells were stored at −20 °C until used. The hybridoma supernatants diluted 1:1 in PBS were incubated for 1 h with the infected and the non-infected cultures, washed and incubated for 30 min with fluorescein isothiocyanate-conjugated rabbit antiserum to mouse immunoglobulins (Dakopatts, Copenhagen, Denmark) and observed with a fluorescence microscope.

Neutralization tests

For initial screening of neutralizing MAbs, 100 tissue culture infectious doses ($TCID_{50}$) of virus in 50 µl of culture medium were incubated with 50 µl of supernatants from hybridomas for 2 h at 14 °C. Then 50 µl of the mixture was transferred to 96-well plates containing monolayer cultures of EPC cells. Cultures were incubated at 14 °C and examined daily for cytopathic effect. To confirm the presence of neutralizing activity, plaque neutralization tests were used in 24-well plates by using overlays of 0·8% ultra-low gelling temperature agarose (Sigma Chemical Co., St Louis, MO, USA). To calculate neutralization indexes (Rovozzo & Burke 1973), the ammonium sulphate concentrates of MAbs were incubated at 4 °C overnight with serial 10-fold dilutions (10^{-2} to 10^{-7}) of 10^5 $TCID_{50}$ ml^{-1} suspensions of virus in tetraplicates. Then 100 µl of the mixture was added to EPC monolayers in 96-well cultures (200 µl final volume). Other details were as shown in Table 3.

Results

Characterization of MAbs

After 3 months of immunization, the ascites from the immunized mouse contained polyclonal antibodies against the viral proteins G, N, Nx and M_1 and did not neutralize the VHSV infectivity. After 9 months of immunization, the ascites from mouse containing polyclonal antibodies against VHSV reacted with viral proteins G, N, Nx, M_1 and M_2 (Fig. 1) and neutralized the

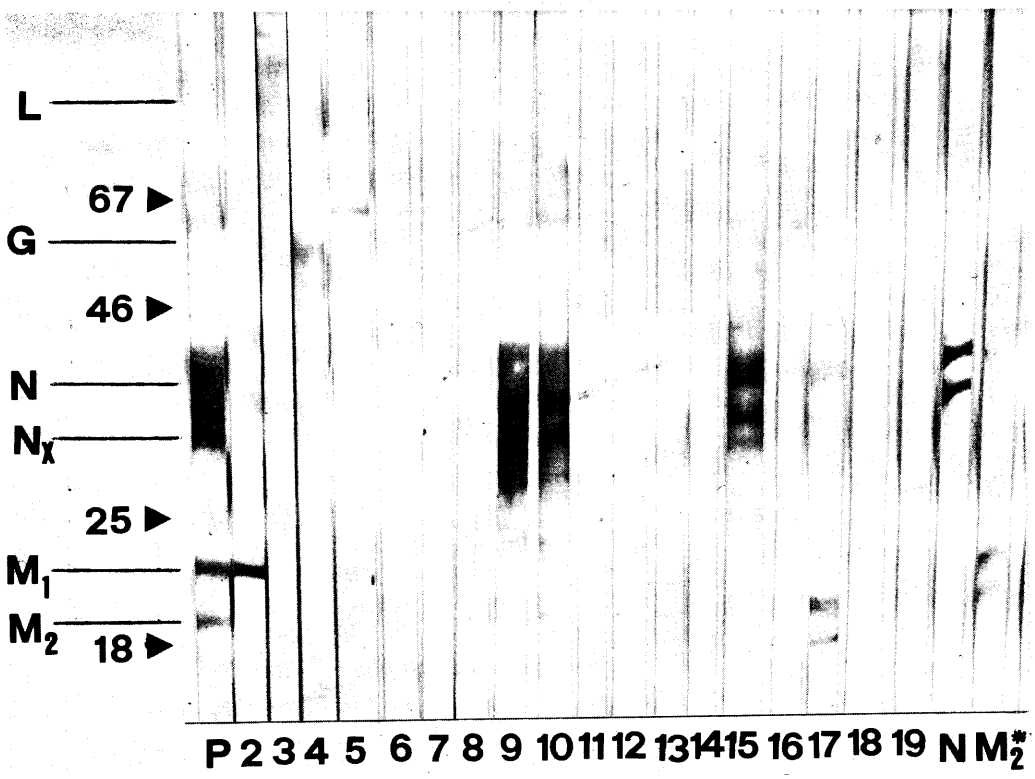

Figure 1. Immunoblotting of MAbs to PEG-concentrated VHSV. PEG-concentrated VHSV was electrophoresed in a 10–15% polyacrylamide gel gradient and transferred to nitrocellulose. The right border of the gel ran somewhat shorter than the left and a band at <18 kDa marker was detected in most of the blots. The strips of nitrocellulose were incubated with hybridoma supernatants and developed as indicated. Viral proteins (letters to the left) were identified by comparison of [^{35}S]-labelled, viral-induced proteins from infected cells with non-infected controls (Nx was characterized as viral-induced protein, Basurco *et al.* 1991), Coomassie-blue staining of the gel and Amidoblack staining of the nitrocellulose filter. P, polyclonal ascites anti-VHSV raised in mice; 2–19, supernatants from anti-VHSV selected hybridomas, as indicated in Table 1; N* and M_2*, MAbs gift of Dr Vestergard-Jorgensen. The numbers to the left are the molecular weight markers run in parallel. MAb 1E3 number 1 (Table 1) has not been included in this figure, since it did not produce any bands.

VHSV infectivity. A total of 19 hybridomas, presumably secreting antibodies to VHSV as measured by indirect ELISA, were isolated from half of the fusion. Table 1 shows the results of immunofluorescence, immunoblotting and neutralization tests of the MAbs selected by indirect ELISA.

By immunofluorescence, five MAbs were clearly positive. MAbs against the M_1 protein gave a strong, coarsely granular staining, primarily in the peripheral part of the cytoplasm. MAb 2C9 against the N caused a fine staining throughout the cytoplasm as seen by the immunoperoxidase technique.

The target antigens of the MAbs were identified by immunoblotting against electrophoretically separated proteins from PEG-concentrated VHSV so that the Nx component was present (Basurco *et al.* 1991). Figure 1 shows the results of immunoblotting. The bands were identified as viral induced G, N, Nx, M_1 and M_2 by comparison of [^{35}S]-labelled protein patterns from infected cells with non-infected controls. Of the 19 MAbs selected by indirect ELISA, only seven were capable of immunologic binding to these denatured proteins. None of these MAbs

Table 1. Characteristics of MAbs selected by indirect ELISA from half of the fusion*

Clone	Number in Fig. 1	Isotype	ELISA	IF	Blotting	Neutralization
1E3	1	IgM K	+	+−	−	−
1C10	2	IgG_1 K	+	+	M_1	−
1E4	3	IgG_3 K	+	+−	L?	+−
1H10	4	IgG_1 K	+	+−	G	+
1F10	5	IgG_{2b} K	+	−	G?	−
1G12	6	ND	+−	−	−	−
1B10	7	ND	+	−	−	−
1D1	8	ND	+−	−	−	−
2D5	9	IgG_1 K	+	+	N + Nx	−
2C9	10	IgG_{2a} K	+	+	N + Nx	−
2B3	11	IgM K	+	−	−	−
2G1	12	ND	+−	+−	−	−
2D2	13	ND	+−	−	−	−
3F10	14	ND	+	+−	−	−
3F7	15	IgG_1 K	+	+	N + Nx	−
3A8	16	ND	+	−	−	−
4E4	17	IgM K	+	+	M_2	−
4E10	18	ND	+	+−	−	−
4D12	19	ND	+	+−	−	−
Polyclonal		−	+	+	G,N,Nx,M_1,M_2	+

* IF, immunofluorescence. The isotype was determined by ELISA with a Biorad Kit. The results of ELISA were classified as positive (+) when the A492 nm was ≥0·3 in the virus-coated well, provided that the A492 nm of the corresponding well coated with non-infected EPC cells did not exceed 50% of the value with virus antigen; otherwise, they were considered negative or doubtful (±). The results of immunofluorescence were classified either as positive (+) when infected cells gave immunofluorescence, and non-infected cells gave no immunofluorescence or as doubtful (±) when some immunofluorescence was seen in non-infected cells. Blotting results are described in Fig. 1. Neutralization was as described in 'Materials and methods'. ND, not determined.

reacted with a cellular control antigen. The anti-G MAbs were preliminarily identified by taking into account that the G molecular weight varies between 90 kDa when glycosylated (Deuter & Enzmann 1986) to 55 kDa when fully deglycosylated (Lorenzen *et al.* 1990). MAb 1H10 reacted with a 58 kDa component coincident with the Coomassie-blue-stained band identified as G, whereas MAb 1F10 reacted with a minor component at 65 kDa. It appears that anti-M_2 MAb reacted slightly with the N protein and an unidentified non-specific component of about 17 kDa; in addition, some anti-N reacted with the M_2 protein (especially 2C9). All the MAbs reacting with the N protein recognized also the Nx protein (Basurco *et al.* 1991) (Fig. 1). Furthermore, the four anti-N MAbs recognized to the same extent highly purified virus (0·5% Nx and 99·5% of N) and PEG-concentrated viral preparation (about 40% of Nx and 60% of N) for each of the five Spanish isolates and the three reference strains of VHSV. Although anti-M_2 MAbs (4E4 and M_2*) seemed to recognize another band at a lower molecular weight, this band was present throughout the gel and was probably an artefact. The MAbs clearly identified by immunoblotting were anti-M_1 (1C10), -G (1H10), -N/Nx (2D5, 2C9 and 3E7) and -M_2 (4E4). MAb 1F10 has been tentatively classified as anti-G.

To confirm the immunoblotting results, G, N, Nx, M_1 and M_2 proteins were isolated by electroelution from preparative electrophoresis. Purity, as estimated by densitometry of the silver-nitrate-stained gels following re-electrophoresis of the isolated proteins, was greater than 80%. The ELISA performed with the isolated viral proteins coated onto the solid-phase and

the MAbs obtained as ascites, confirmed most of the identifications by immunoblotting (Table 2), although the anti-G 1F10 results were not clear. MAb anti-N 2D5 also recognized M_1 to some extent, most probably due to their higher titre. Specificity of 1H10 was further confirmed by ELISA over plates coated with non-denatured purified protein G by another laboratory (about 2 ng well^{-1} sensitivity).

Neutralizing MAb

Neutralization tests performed during the screening of 500 hybridomas gave non-reproducible results depending on the relative amounts of the hybridoma culture supernatants and the VHSV. Therefore, the present authors studied neutralization by plaque reduction with the two anti-G MAbs (1H10 and 1F10) described in Table 1. Figure 2 shows that most of the supernatants from the hybridomas tested did not change the number of plaques, except those from 1H10 which showed a two-fold increase and the polyclonal ascites which halved the number of plaques. Neutralizing antibodies might increase the number of plaques when used at subneutralizing concentrations (Peiris, Gordon, Ukeless & Porterfield 1981; Bolognesi 1989; Burstin, Brandriss & Schlsinger 1983), and therefore, the present authors concentrated both MAbs and polyclonal Abs. After increasing their concentration to 300 μg ml^{-1} in plaque reduction tests, total neutralization of VHSV was obtained in both cases (Table 3). Under the experimental conditions used, neutralization indices of 0, 1·8, 1·7 and 1·2 were obtained for IHNV, VHSV-F_1, VHSV-F_2 and VHSV-23·75, respectively. Under the same experimental conditions, anti-IHNV MAb 5G3 (gift of Dr Winton) showed a neutralization index of 2·5 against IHNV (Table 3).

Variability of epitopes by ELISA

Table 4 shows the reactivity of five VHSV Spanish isolates, three VHSV reference serotypes (as defined by seroneutralization) and one IHNV isolate with nine MAbs (seven of them selected

Table 2. Reaction of MAbs with VHSV proteins purified by electroelution*

	VHSV electroeluted proteins				
MAbs (EI)	G	N	Nx	M_1	M_2
1H10 (G)	0·3	–	–	0·12	–
1F10 (G?)	0·16	–	–	–	–
2D5 (N)	0·22	0·75	0·65	0·21	–
2C9 (N)	–	1·05	0·75	–	–
3E7 (N)	–	0·90	0·75	–	–
1C10 (M_1)	0·11	0·06	–	1·00	–
4E4 (M_2)	0·11	0·02	–	0·15	0·65

* The ELISA assays were performed by using 0·5 μg viral protein well^{-1}. The MAbs were obtained as mouse ascites and tested at serial five-fold dilutions (50–6250-fold dilutions) so as to obtain comparable results. Results are expressed as absorbances at 492 nm. Background obtained by using an irrelevant mouse ascites and by each MAb over non-coated wells was subtracted from all the data. EI, identification of bands as estimated by immunoblotting. –, ≤ background.

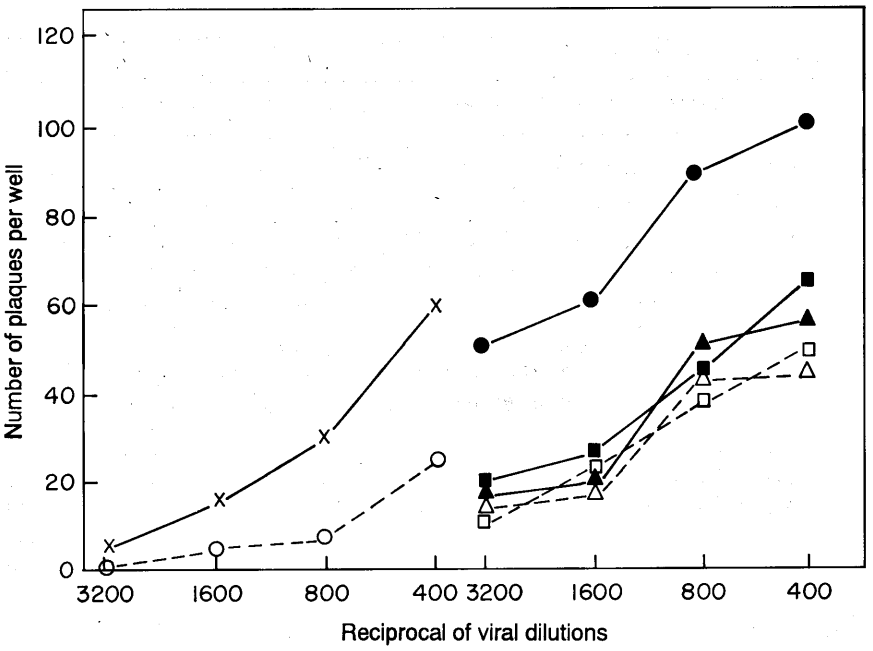

Figure 2. Number of plaques obtained after incubating VHSV with hybridoma supernatants against VHSV proteins. VHSV at 8×10^4 PFU ml^{-1} was assayed in 24-well plates at different dilutions in the absence or presence of MAbs from supernatants of cloned hybridomas, as selected by ELISA and identified by immunoblotting. Virus (300 μl) and MAbs (100 μl) were mixed and incubated at 14 °C overnight, then absorbed to the EPC monolayers at 14 °C for 1 h, washed, overlayed with 400 μl of 0·8% low-melting point agarose per well and incubated at 14 °C for 4–5 days. (X—X) no Abs; (○—○) mouse ascites PAbs; (●—●) supernatant from 1H10 (G); (■—■) supernatant from 1C10 (M_1); (□—□) supernatant from 1E4; (▲—▲) supernatant from 2C9 (N); (△—△) supernatant from 1F10.

Table 3. Neutralization of VHSV serotypes and IHNV*

	Antibodies to:			
Viruses	IHNV 5G3	VHSV Polyclonal	VHSV 1F10	VHSV 1H10
IHNV – Cedar	2·5	N.D.	−0·5	0
VHSV – F_1	−0·2	0·8	0·3	1·8
VHSV – F_2	−0·2	0·5	0	1·7
VHSV – 23·75	−0·2	1·2	0	1·2

* All the antibodies used were from mouse ascites concentrated by ammonium sulphate except the anti-IHNV which was used as concentrated supernatants from hybridoma 5G3 (gift of Dr Winton). To calculate neutralization indexes, the ammonium sulphate concentrates were incubated at 4 °C overnight with serial 10-fold virus dilutions (10^{-2} to 10^{-7}) of 10^5 $TCID_{50}$ ml^{-1} suspensions in tetraplicates at a final concentration of 300 $\mu g\,ml^{-1}$ in culture medium. Then 100 μl of the mixture was added to EPC monolayers in 96-well cultures (200 μl total final volume) and plates incubated for 3 days at 14 °C. Neutralization index = −log ($TCID_{50}$ of virus control − $TCID_{50}$ of tested MAb) (Rovozzo & Burke 1973). ND, not determined.

Table 4. Variability of VHSV from Spanish isolates (Basurco & Coll 1989), VHSV serotypes and IHNV as studied with anti-VHSV MAbs†

	Viruses								
MAbs	798	471	472	689	144	F_1	F_2	23·75	IHN
G? 1F10	1·6	0·8	0·7	0·6	1·8	0·6	1·0	1·0	0·6
G 1H10	1·2	0·7	0·6	0·9	1·2	0·6	0·8	1·0	0·4
N 2D5	2·0	2·2	2·0	1·9	1·8	2·2	1·9	1·8	0·2
N 2C9	2·4	2·5	2·0	2·2	2·2	2·2	2·0	2·2	0·2
N 3E7	2·2	2·3	1·9	2·1	2·1	1·9	2·0	2·0	0·2
N*	2·0	1·8	1·8	1·8	1·8	1·3	1·6	1·5	0·1
M_1 1C10	2·6	0·3	0·5	1·3	1·6	1·3	1·0	2·2	0·2
M_2 4E4	3·2	2·7	2·0	1·7	2·7	0·4	1·7	2·8	0·6
M_2*	1·6	1·7	1·7	1·6	1·2	1·4	1·3	1·2	0·1

† Spanish virus isolates were from *Barbus graellsi* (798), *Oncorhynchus mykiss* (471, 689, 144) and *Salmo salar* (472). The ELISA assays were performed by using 0·3 μg purified virus well^{-1}, except those for G MAbs (3 μg well^{-1}) and the IHNV (1 μg well^{-1}). The supernatants of the hybridomas were diluted 20-fold in dilution buffer so as to produce an ELISA titre of the same order of magnitude. The results were finally normalized by the ELISA absorbances in parallel by the use of rabbit polyvalent antisera ($F_1 + F_2 + 23·75$) used as international reference standard (gift of Dr de Kinkelin). The correction factor varied between one- and two-fold. The results were similar whether rabbit anti-VHSV 144 antisera or mouse anti-VHSV ascites were used for normalization. Background values were negligible. MAbs as shown in Fig. 1 and *as Lorenzen *et al.* (1988).

in this work plus two gifted by Dr Vestergard-Jorgensen). The MAbs studied (especially the anti-N) reacted similarly with most of the VHSV isolates. However, no reactions were obtained between the anti-M_1 (1C10) and the Spanish isolates 471 (*O. mykiss*) and 472 (*S. sālar*), between the anti-M_2 (4E4) and the serotype F_1, and between the anti-N (2C9), -N (3E7), -*N and -*M_2 and the IHNV. The MAb anti-G region (1F10), −G (1H10) and −M_2 (4E4) crossreacted slightly with the IHNV (Cedar Strain) used in this study. No strong species specific differences were noted in the reactivity of the isolates taken from the different fish species (*B. graellsi* 798; *O. mykiss* 471, 689, 144; *Salmo salar* 472) with the panel of MAbs.

Discussion

Important steps in obtaining MAbs against the proteins of VHSV were the development of a highly sensitive ELISA and a long-term immunization protocol. The present authors used coating to dryness which increased the amount of virus in the solid-phase (about 10-fold), and thus, increased the sensitivity. To increase the probability of obtaining anti-G MAbs with neutralizing activity, mice were immunized for 9 months until their ascites fluid contained both neutralizing activity and antibodies against most of the viral proteins (Table 1 & Fig. 1).

The present authors found that all the MAbs tested against the N protein, recognized the Nx protein as described previously (Basurco *et al.* 1991). In contrast, to the studies of Lorenzen *et al.* (1988) and Bernard, Lecocq-Xhonneux, Rossius, Thiry & de Kinkelin (1990), where there was only a small amount of Nx in the purified virus, the present authors have shown that Nx is a major component of concentrated virus (complete virus and free nucleocapsids). This observation has been made by Coomassie-blue staining, [^{35}S]-methionine labelling and immunoprecipitation of the five Spanish VHSV isolates (1984–1986) and the reference VHSV strains (F_1, F_2, 23·75) (Basurco & Coll 1989a, b).

The present authors also observed cross-reaction of anti-M_2 MAbs with the N band, as reported by Lorenzen *et al.* (1988). By using ELISA, several VHSV isolates were tested against the panel of MAbs. The present authors were only able to distinguish isolates 471 and 472 from the rest by their lack of reactivity with anti-M_1 (1C10). Isolates 471 and 472 came into the laboratory during the same week in 1986, although from two different Spanish locations.

Because the identification of fish rhabdoviruses by conventional neutralization tests is often hampered by the difficulty of producing high titre sera to neutralize them, one of the purposes of this study was to develop neutralizing MAbs against VHSV. However, only a paradoxical increase in VHSV infectivity in the presence of low concentrations of one anti-G Mab was found (Fig. 2). To obtain neutralization, this MAb had to be used at a very high concentration (Table 3). Similar results have been described for many other enveloped viruses (Peiris *et al.* 1981; Burstin *et al.* 1983; Bolognesi 1989), including another non-salmonid fish rhabdovirus (Clerx, Horzinek & Osterhans 1978).

Why are the fish rhabdoviruses so resistant to *in vitro* neutralization? On the one hand, this could be due to the low titre of the antibodies because of the G epitopes instability. For instance, some G epitopes are dependent on intact disulphide bridges (Leong 1990; Lorenzen *et al.* 1990), the virus is easily inactivated by moderately high temperatures (de Kinkelin, Berre & Bernard 1980; Vestergard-Jorgensen 1982) and the G-containing spikes can be easily stripped-off during the purification (de Kinkelin 1972; Olberding & Frost 1975). On the other hand, the difficulty could be due to the high particle to infectivity ratio (Hsu & Leong 1985). Thus, the apparent neutralizing activity of a reagent could be determined not only by the concentration of neutralizing antibody, but also by the proportion of viral particles and components present in the infective material. The binding of antibodies to non-infective viral particles could interfere with the neutralization of the infective viruses. At subneutralizing concentrations, the antibodies would eliminate the non-infective particles favouring the infection of the infective virus (enhancement effect). The low titre of Abs, the high number of non-infective viruses, plus their possible complement dependence (Clerx *et al.* 1978) could be all interfering in the *in vitro* neutralization results. Finally, the neutralizing activity as defined by the *in vitro* neutralization assays might not be totally indicative of the *in vivo* mechanism in the trout, since some *in vivo* neutralizing (passive immunization) anti-VHSV MAbs do not have neutralizing activity *in vitro* (Lorenzen *et al.* 1990).

Whereas the use of MAb to analyse four rabies serotypes (Dietzschold, Tollis, Rupprecht, Celis & Koprowski 1987) has demonstrated antigenic variability among different isolates (Flamand, Wiktor & Koprowski 1980), the present authors did not find variability in the VHSV epitope defined by neutralizing MAb (1H10). However, this could be due to its low neutralizing specific activity. MAb 1H10 is unique in that it has some neutralizing activity, yet it recognizes fully denatured G protein contrary to other MAbs with neutralizing VHSV activity (Lorenzen *et al.* 1990). Until more neutralizing MAbs to VHSV are made available worldwide, a complete study of the epitope variability of the G protein will remain wanting.

In addition to being useful in basic VHSV studies, the MAbs developed during this work could be useful for neutralization, ELISA and immunofluorescence. Of the 19 MAbs selected by ELISA, 12 were specific for the virus by immunofluorescence, and six were further identified by ELISA and confirmed by immunoblotting, as follows: three against the N protein, one against M_1, one against M_2, and one against G. Five MAbs were found suitable for immunofluorescence, one for immunoperoxidase, and the ones against the N protein have been very useful for developing an ELISA due to their higher titre, recognition of the three VHSV serotypes and specificity for the earliest major virus-induced protein.

Acknowledgments

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