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DETECTION OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS BY DIRECT IMUNOPEROXIDASE WITH SELECTED ANTI-NUCLEOPROTEIN MONOCLONAL ANTIBODY

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Introduction

The viral haemorrhagic septicaemia virus (VHSV) is a membrane enclosed, negative strand RNA virus which buds from the infected cell membrane. The VHS is a viral disease of salmonid fishes causing severe damage in fish farms in Europe (de Kinkelin, 1972). The five agreed-upon virion proteins of VHSV are designated by the letters, L (the RNA-dependent RNA polymerase of 150-200 KDa), N (the majoritary phosphorylated nucleoprotein of 45-50 KDa), Nx (a protein antigenically related to N, (Basurco et al, 1991), Ml and M2 (the matrix proteins of 22-28 KDa) and G (the neutralizing epitope-carrier glycoprotein of 60-80 KDa) (Deuter & Enzmann, 1986).

In the experiments reported here, we selected a suitable MAb from a panel of anti-N MAbs to perform the technique of the immunoperoxidase for rapid diagnostic tests over infected cell culture monolayers.

Materials and Methods

Viruses.- The strains of virus used were VHSV-Fl and VHSV-F from Dr. P.E. Vestergaard-Jorgensen. VSHV-23.75 from Dr. P.de Kinkelin and IHNV-Cedar strain from Dr. R. Hedrick. Five VHSV isolates were obtained from fish tissue samples in Spain. The virus were isolated from: rainbow trout, *Oncohrynchus 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) (Basurco & Coll, 1989a,b; Basurco, 1990).

Cells, media and virus purification.- Epithelioma papillosum cyprine (EPC) cell culture techniques (RPMI-1640 medium, Flow, Ayrshire, Scotland) and virus purification were essentially as reported by de Kinkelin (1972) and modified by Basurco & Coll (1989, b).

Anti-VHSV Monoclonal antibodies (MAbs).- We followed techniques described previously (Martinez & Coll, 1988). Briefly, female mice (BALB/c) were given 9 intraperitoneal injections of 25µg of VHSV protein over a period of 9 months. Spleen cells from the immunized mice were fused with the myeloma cell line P3-X63-Ag 8653. Fusion, cloning twice by limiting dilution indirect ELISA, cultivation of hybridoma cells and ascites production were performed as described previously (Lorenzen *et al*, 1988; Basurco *et al*. 1991).

Indirect ELISA. - This was performed by following previously described methods (Martínez and Coll, 1987,1988). Briefly the MAb and their conjugates were titrated by ELISA on VHSV-coated microtitration plate wells. Then 0.3µg of purified VHSV were added to the wells of polystyrene plates in 100µl of distilled water, dried overnight at 37 °C, and then washed with washing buffer. The MAb were diluted in dilution buffer (130mM NaCl, 2mM KCl, 8mM Na_2 HPO_4, 1.4 mM KH_2PO_4, 0.24mM merthiolate, 5g Tween 20/l, 50mg phenol red/l, pH 6.8; ten-fold-diluted with distilled water, this was used as the washing buffer) and 100µl was added to the wells. After 1h incubation and washing, 100µl of peroxidase-labelled anti-mouse IgG (Nordic, Tilburg, The Netherlands) was added and incubation continued for 30 minutes until color development.

For color development, 50µl of substrate buffer (150mM sodium citrate, 3mMH_2O_2 and 1mg o-phenylenediamine/l, pH 4.8) was added, and the reaction was stopped by addition of 50µl of 4M H_2SO_4 after 30 min-

utes. Results were read at 3 wavelengths 450, 492 and 620nm. Absorbance at 620 nm was used to correct for individual non-significant differences between wells. Absorbance at 450nm was used to calculate the 492nm absorbance values ≥2 by aproximation (absorbance at 492nm= absorbance at 450nm x 2.4).

Indirect immunofluorescence.- Cover-glass cultures of EPC cells were infected with 10^6TCID$_{50}$ per ml of VHSV Spanish isolate 144 (Basurco and Coll, 1989a) and incubated at 14°C for 1-2 days. After washing with medium without serum, the cultures were fixed for 10min in acetone. These fixed cells were stored at -20°C until used. The hybridoma supernatants diluted 1:1 in PBS were incubated for 1h with infected and noninfected cultures, washed and incubated for 30 min with fluorescein isothiocyanate-conjugated rabbit antiserum to mouse immunoglobulins (Dakopatts, Copenhagen) and observed with a fluorescence microscope.

Direct imunoperoxidase.- Mouse ascites from the producer hybridoma of MAb 2C9 was obtained by injection of physiological saline as described (Coll, 1987). The ascites were clarified by low speed centrifugation and stored at -40°C until use. The ascites were purified by affinity chromatography over Protein A-Sepharose columns (Pharmacia, Uppsala, Sweden). Sample buffer was 1.5M glycine, 3M NaCl, pH8.9. The retained MAb 2C9 (IgG_{2a}) was eluted with 0.1M citric acid, pH4.9. Eluted fractions were pooled and dialized against 10mM sodium phosphate, 150mM NaCl, pH7.2 (PBS). Purity as tested by gel electrophoresis gave two single Coomassie blue stained bands at 50 and 24KDa, respectively. To couple peroxidase to MAb 2C9 by the one-step glutaraldehyde method, 0.7mg of MAb was mixed with 20mg of horseradish peroxidase, E.C. 1.11.1.7., 1000 U/mg, RZ/3.3 (Boehringer Mannheim) in 0.4 ml of PBS pH7.2 and 10µl of 25% glutaraldehyde. After incubation at 37°C for 2 h, 400µl of 1M glycine were added and incubated overnight at room temperature. The resulting conjugates had a molar ratio of antibody to peroxidase of 0.9 to 1. EPC cultures were prepared and stained with a 50-fold dilution of conjugate, other details were as for immunofluorescence (see above), but after incubation with the conjugate, the slides were washed with PBS and incubated for 15 min with 1mg/ml diaminobenzidine in low background citrate buffer (Coll,1989).

Results

A total of 19 hybridomas, presumed to be secreting antibodies to VHSV as measured by indirect ELISA, were studied. Three anti-N MAbs were selected from the panel of anti-VHSV MAbs by immunoblotting and confirmed by ELISA over purified protein N.

Table 1 shows the reactivity of 5 VHSV Spanish isolates, 3 VHSV reference serotypes (as defined by seroneutralization) and 1 IHNV with the 3 selected MAb. No strong differences were recognised in reactivity with the panel of MAbs amongst the isolates studied. All 3 MAbs (2D5, 2C9 and 3E7) reacting with the N protein also recognized the Nx protein (Basurco et al, 1991). Furthermore, the 3 MAbs anti-N recognized to the same extent highly purified virus (0.5% Nx and 99.5% of N) and PEG-concentrated viral preparations (about 40 % of Nx and 60% of N) for each of the 5 Spanish isolates and the 3 reference strains of the VHSV (data not shown).

The 3 MAbs were obtained as mouse ascites, purified by affinity chromatography over protein-A and titrated by indirect ELISA. Titres were 1/5,000 for 2D5 (IgG_1K), 1/6,000 for 3E7 (IgG_1K) and 1/31,000 for

Table 1. Variability of VHSV from Spanish isolates (Basurco and Coll, 1989), VHSV serotypes and IHNV as investigated with MAbs anti-VHSV nucleoprotein.

	Viruses								
MAbs	798	471	472	689	144	F1	F2	23.75	IHN
2D5	2.0	2.2	2.0	1.9	1.8	2.2	1.9	1.8	0.2
2C9	2.4	2.5	2.0	2.2	2.2	2.2	2.0	2.2	0.2
3E7	2.2	2.3	1.9	2.1	2.1	1.9	2.0	2.0	0.2

The ELISA assays were performed by using 0.3µg of purified VHSV per well except those for the IHNV (1µg/well). The supernatants of the hybridomas were diluted 20-fold in dilution buffer so as to produce an indirect ELISA titre of the same order of magnitude. Background values were < 0.2

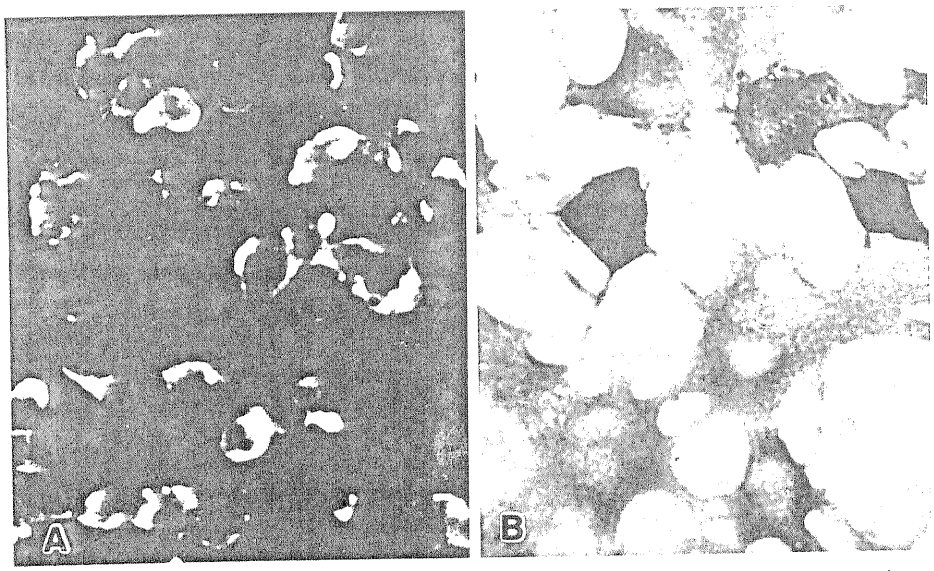

Figure 1.- VHSV-infected EPC cells stained by immunofluoresence (A) or by direct immunoperoxidase (B) with MAb 2C9.

2C9 ($IgG_{2a}K$). Peroxidase conjugates optimized by using glutaraldehyde for different lengths of time allowed the selection of 2C9 as the best MAb for direct immunoperoxidase techniques.

By immunofluorescence, five MAbs were clearly positive. MAbs against the N protein stained strongly by immunofluorescence, primarily at the peripheral part of the cytoplasm (Fig 1A). MAb 2C9 against the N resulted in strong staining throughout the cytoplasm as seen by the direct immunoperoxidase technique (Fig. 1B).

Discussion

Anti-N MAbs were selected to optimize the immunoperoxidase assay because the N protein is the less variable of the rhabdoviruses in general so that this assay could serve to detect the greatest number of viral serotypes and isolates. The epitopes defined by the anti-MAbs chosen were highly conserved in the 3 defined VHSV serotypes and in the Spanish isolates (Table 1). The N protein of VHS is a majority component found both in the complete virus and in infected cells and the N protein is the first VHSV protein synthesized 1-2 h after infection.

Whilst the use of anti-N MAbs to analyze rabies have demonstrated antigenic variability amongst different isolates (Flamand et al, 1980), we did not find variability in the VHSV epitopes defined by the anti-N MAbs used. Due to their higher titre, the anti VHSV MAbs against the N protein have been very useful for development of direct immunoperoxidase technique which provides some advantages over the immunofluorescence method. These advantages include the elimination of background staining, the use of an ordinary light microscope and the possibility of keeping the result for a long time. However, only when the virus titres of frozen samples of kidney and spleen of infected fish were $>10^8$ $TCID_{50}/g$ did they show a positive reaction (Faisal and Ahne, 1980). Because of their low sensitivity and because of the need to process samples separately, this technique is not very useful for the detection of carriers, but field samples from VHSV outbreaks can now be assayed with a high degree of specificity and in a short period of time from infection of cell culture monolayers.

Summary

Three viral haemorrhagic septicaemia virus (VHSV) international reference serotypes (Fl, F2,and 23.75) and five VHSV isolates from different host species (trout, salmon and barbel) and spanish geographical locations, were recognized by the anti-N monoclonal antibody (MAb) 2C9. The high titre and specificity of this MAb favour it to be conjugated to peroxidase by the one step glutaraldehyde method making possible its use in the direct immunoperoxidase cytological techniques over EPC infected monolayers.The reagent obtained will improve diagnostic tests currently using polyclonal antibodies.

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