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Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus

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ABSTRACT: The preparation of the first monoclonal antibody (MAb) against the orthomyxovirus-like infectious salmon anaemia (ISA) virus is described. Characterization of the MAb included isotyping, enzyme-linked immunosorbent assay (ELISA), immunofluorescent staining of virus infected cell cultures (SHK-1 cells), immunoelectron microscopy (IEM) of negatively stained virus preparations, virus neutralization assay and haemagglutination inhibition assay. The MAb reacted with ISA virus preparations both with immunofluorescent staining and in ELISA. No reactions were observed in cell cultures infected with other viruses infecting salmonids including infectious pancreatic necrosis (IPN) virus, viral haemorrhagic septicaemia (VHS) virus and infectious haematopoietic necrosis (IHN) virus. The MAb was also shown to neutralize ISA virus infection in cell cultures and to inhibit the haemagglutination reaction. IEM demonstrated binding to the surface of negatively stained ISA virions. Thus, it is concluded that the MAb binds to the haemagglutinin on the virion surface. Furthermore, using immunofluorescent staining of virus infected cell cultures, reactivity against all the 13 ISA virus strains currently available was demonstrated. Using the MAb, a simple, rapid direct immunofluorescent assay for ISA virus detection and titration in 96-well tissue culture plates was developed. Infectivity titrations by this method correlated well with titration by cytopathic effects. The reliability of the assay was demonstrated by close agreement in virus infectivity titres among different assays for the same virus that were performed on the same day and on different days. A method for detection of viral antigen in cryosections from ISA diseased fish is also reported that may prove useful for the diagnosis and control of ISA.

KEY WORDS: Infectious salmon anaemia virus · Monoclonal antibodies · Infectivity titration · Immunofluorescence

INTRODUCTION

Infectious salmon anaemia (ISA) is a disease of considerable economic importance for the farming of Atlantic salmon *Salmo salar* L. in Norway. The disease is caused by an orthomyxo-like virus (Dannevig et al. 1995, Falk et al. 1997, Mjaaland et al. 1997) and pathological changes are characterized by severe anaemia, leucopenia, congestion of the liver, spleen and foregut, haemorrhagic liver necrosis, petechiae in the viscera, and ascites (Thorud & Djupvik 1988, Evensen et al. 1991, Thorud 1991). The disease spreads slowly through an affected farm and accumulated mortality rates after several months range from 15 to 100% (Thorud 1991).

Determination of the prevalence and the effect of ISA on salmon farming in Norway is difficult because present diagnostic methods are limited to the evaluation of clinical appearance, gross changes, histologic examination and haematological findings. The sensitivity and specificity of these diagnostic methods are unknown and these methods are unlikely to be useful for determining the prevalence of ISA virus. It has also been difficult to diagnose unequivocally early or mild cases of ISA in fish with minor pathological lesions or in fish with multiple diseases. Accordingly, there has been a need to develop more specific laboratory methods for the detection of ISA virus.

In the present study, the production and characterization of a monoclonal antibody (MAb) against the ISA virus is described. The MAb was used to develop a

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simple, rapid direct immunofluorescent assay (IF) for ISA virus detection and titration in 96-well tissue culture plates. The detection of viral antigen in tissue cryosections using the MAb and indirect immunofluorescence (IIF) is also described. A preliminary report of this work was presented at the Third International Symposium on Viruses of Lower Vertebrates (Falk & Dannevig 1995).

MATERIALS AND METHODS

Virus and cell culture. In this study, the ISA virus strain isolated by Dannevig et al. (Dannevig et al. 1995), now designated strain Glesvaer/2/90, was used. All assays were performed with fourth to sixth passage of the virus. Cultures of SHK-1 cells (Dannevig et al. 1995) were used for virus propagation. The cells were grown at 20°C in Leibovitz L-15 medium supplemented with 5% foetal bovine serum, glutamine (4 mM), 2-mercaptoethanol (40 µM) and gentamicin (50 µg ml^{-1}) (L-15). Cells were incubated at 15°C after inoculation with virus. Unless otherwise stated, cells with fluid overlay removed were inoculated with virus at a multiplicity of infection (MOI) of 0.1 in serum-free L-15. After virus adsorption for 4 h at 15°C, the inoculum was removed and fresh L-15 was added.

New ISA virus strains were isolated from tissue homogenates (10%, w/v in serum-free culture medium) of kidney tissue from naturally infected Atlantic salmon as previously described (Dannevig et al. 1995). Geographic location by county (in Norway), year of ISA outbreak, and designation of the various strains are given in Table 1 in 'Results'.

Production of hybridomas. Antigen for immunization of mice was prepared from cell culture medium harvested 4 d after inoculation with the virus (i.e. at the time when the first sign of cytopathic effects [CPE] appeared). Briefly, the culture medium was clarified by centrifugation (3000 × *g*, 30 min) followed by pelleting at 100 000 × *g* for 1 h. The pellet was resuspended in phosphate buffered saline (PBS; 10 mM, pH 7.4), giving approximately a 70-fold concentration of virus. Female BALB/c mice were given a subcutaneous injection of 0.1 ml of the concentrated virus mixed 1:1 with Freund's complete adjuvant followed by 2 injections, 2 wk apart, using Freund's incomplete adjuvant. A final intraperitoneal booster was given 4 d prior to harvesting of spleen cells. Serum was collected from the mice and tested for antibody production by enzyme-linked immunosorbent assay (ELISA) and IIF as described below.

Hybridoma cultures were produced by standard methods (Köhler & Milstein 1975). Spleen cells were collected from immunized mice and fused with SP2/0-Ag-14 cells (Shulman et al. 1978) at a ratio of 4:1 in polyethylene glycol 1500 (Boehringer Mannheim, Mannheim, Germany). The fused cells were resuspended in growth medium (GM) consisting of Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 IU ml^{-1}) and streptomycin (0.1 mg ml^{-1}) supplemented with 20% foetal bovine serum and 5% human endothelial culture supernatant (HECS; Costar, Cambridge, MA, USA), and distributed to ten 96-well culture plates (100 µl $well^{-1}$) and incubated at 37°C in a humidified environment with 5% CO_2. After 24 h, 100 µl $well^{-1}$ of GM supplemented with hypoxanthine, aminopterin and thymidine (GM-HAT) to give final concentrations of 0.1 mM, 4×10^{-4} mM, and 1.6×10^{-2} mM, respectively, was added. After 1 wk, medium was replaced by fresh GM-HAT, and after 2 wk medium was replaced by GM-HT.

Hybridomas producing antibody were cloned twice by the limited dilution method in 96-well culture plates and adapted to HT- and HECS-free GM supplemented with 10% foetal bovine serum. The MAbs used in this investigation were obtained from hybridoma culture supernatants or from mouse ascites. Ascites fluid was obtained from BALB/c mice that received an intraperitoneal inoculation of 2 to 5×10^6 hybridoma cells 1 wk after priming by intraperitoneal injection of 0.5 ml Pristane.

Detection of antibody to ISA virus in mice sera and in hybridoma culture fluids. Culture fluids were screened for antiviral antibody both by ELISA and by IIF. Controls consisted of uninfected SHK-1 cell preparations, as well as culture supernatants from the parental SP2/0-Ag-14 cell line. Serum from the mice was adsorbed by making the working dilution in cell culture medium from a 1 wk old uninfected SHK-1 culture followed by incubation for 30 min at room temperature.

Table 1. Strains of ISA virus obtained and used in this study

Strain designation	Geographic location by county	Year
Meløy/H2143/89	Nordland	1989
Glesvaer/400/90	Hordaland	1990
Glesvaer/2/90	Hordaland	1990
Sotra/2937/91	Hordaland	1991
Sotra/B162/92	Hordaland	1992
Sotra/B797/92	Hordaland	1992
Vestvågøy/2163/93	Nordland	1993
Hinnøy/661/94	Troms	1994
Selje/1525/95	Møre og Romsdal	1995
Hitra/1712/96	Sør-Trøndelag	1996
Vågan/771/96	Nordland	1996
Varaldsøy/768/96	Hordaland	1996
Hitra/1068/97	Sør-Trøndelag	1997

Antigen for ELISA was prepared from infected cells 4 d after inoculation with virus. Infected cells were used because virus did not grow to high enough titers for the use for purified virus in these assays and there was very little background when infected cells were used instead. Cells were washed twice in cold PBS by centrifugation (10 min, 1000 × *g*), scraped off, washed once again and resuspended in a small volume of PBS. Following 3 cycles of freeze-thawing, the antigen preparations was briefly sonicated, aliquoted and stored at –40°C until used. Polystyrene microtitre plates (Immuno-Plate Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 µl well^{-1} of the antigen preparation diluted to 5 µg ml^{-1} in carbonate buffer (50 mM, pH 9.6). The plates were then washed 3 times in PBS with 0.1% Tween 20 (PBS-T) followed by blocking with 5% non-fat dry milk in PBS-T, 150 µl well^{-1}, for 1 h at 37°C. All subsequent incubations were performed at room temperature. The microtitre plates were drained and incubated for 1 h with 50 µl well^{-1} of the hybridoma supernatant to be examined. Serum from the mouse that was used for the fusion served as the positive control. After washing the plates 5 times in PBS-T, 50 µl well^{-1} of peroxidase-conjugated sheep anti-mouse Ig (Amersham, UK), 1/2000 in PBS, was added and the plates were incubated for a further 45 min. Finally, following washing, 100 µl well^{-1} of H_2O_2-OPD substrate (3.5 mM O-phenylendiamine and 0.0012% hydrogen peroxide in 35 mM citrate/150 mM phosphate, pH 5.0) was added. The enzyme reaction was stopped after 20 min by adding 100 µl well^{-1} of 2.3 M H_2SO_4 and the optical density was read at 492 nm. In the initial screening, a hybridoma supernatant was regarded as ELISA-positive when its absorbance value was above 0.3 in the virus-coated well, provided that the absorbance of the corresponding well coated with control antigen did not exceed 50% of the value with virus antigen.

IIF was performed on fixed cell cultures in 96-well culture plates. Cultures were rinsed in PBS, fixed in 80% acetone in distilled water for 20 min, air-dried and stored at 4°C until used. Cultures of non-infected SHK-1 cells were used as controls. After blocking with 5% non-fat dry milk in PBS for 30 min, undiluted supernatants were added and incubated for 1 h. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Southern Biotechnology Associates Inc., Birmingham, AL, USA), 1/100 in PBS, was used for the detection of bound antibodies. PBS-T was used for washing. All incubations were performed at room temperature. The specificity of the staining was confirmed by omitting the primary antibody, or by replacing the anti-ISA virus MAb with an irrelevant IgG_1 MAb. Examination of the cultures was carried out in an inverted epifluorescent microscope (Leitz DMIL) supplied with a 50 W mercury lamp.

Protein determination. The protein concentrations in purified Ig and in virus antigen preparations were measured using a commercial assay (Bio-Rad Laboratories, Richmond, CA, USA) with human gamma globulin or bovine serum albumin (BSA) serving as protein standards.

Isotyping. Monoclonal antibody class and subclass were determined by an ELISA-based mouse Ig Isotyping Kit (Southern Biotechnology Associates Inc.).

Purification and conjugation of antibody. Ascites was clarified by centrifugation (15 min, 3000 × *g*) and purified by affinity chromatography using a Protein G Sepharose FF column (Pharmacia, Uppsala, Sweden) in Na-phosphate buffer (2 mM, pH 7.0). The retained fractions were eluted with glycine buffer (100 mM, pH 2.7) and collected in tubes containing Tris-HCl buffer (1.0 M, pH 9; 40 µl tube^{-1}, 2 ml fractions) to reach a final pH of 7.0. Eluted fractions were then pooled, and dialyzed against PBS. Protein concentration was adjusted to 2.0 mg ml^{-1} following concentration of the pooled fractions in a saturated polyethylene glycol solution. Conjugation of MAbs to FITC followed the procedure given by Goding (Goding 1976). Briefly, purified Ig was dialyzed against carbonate/bicarbonate buffer (1 mM, pH 9.5) overnight. FITC (Sigma, St. Louis, MO, USA) dissolved in dimethyl sulfoxide (10 mg ml^{-1}) was added to 100 µg mg^{-1} Ig and incubated for 3 h at room temperature. Free fluorochrome was separated from the conjugate by chromatography on Sephadex G-25 (Pharmacia) in PBS. The protein concentration of the final conjugate was 1 mg ml^{-1}.

Virus neutralization. The ability of the MAb to neutralize ISA virus was determined using an IF test in 96-well culture plates. Serial 2-fold dilutions of mouse ascites were mixed with an equal volume of tissue culture medium containing 100 $TCID_{50}$ (50% tissue culture infective dose) of virus and incubated for 2 h at room temperature. Fifty µl of the mixture was then transferred to 96-well culture plates containing monolayer cultures of SHK-1 cells in 150 µl well^{-1} culture medium. After incubation for 3 d, the cultures were fixed in 80% acetone in distilled water and stained by the IF test as described below.

Haemagglutination inhibition. Whole blood from Atlantic salmon was collected in Alsevers's solution and washed 3 times in PBS. Haemagglutination was carried out as previously described (Falk et al. 1997). The haemagglutination inhibition (HI) test was performed by first mixing 25 µl of MAb, diluted serially in PBS, with 25 µl virus suspension containing 4 haemaggluting activity (HA) units, followed by incubation for 1 h. Fifty µl of 0.5% Atlantic salmon red blood cells in PBS was then added and the agglutination endpoint was read after 1 h of incubation. All incubations were performed at room temperature. Haemaggluting activ-

ity is expressed as the reciprocal value of the highest dilution showing complete agglutination of the red blood cells.

Immunoelectron microscopy. Immunoelectron microscopy was performed using ISA virus purified by CsCl gradient centrifugation as previously described (Falk et al. 1997). Bromelain treated virions were prepared by mixing equal amounts of purified virus and 4 mg ml^{-1} bromelain (Sigma) in PBS containing 10 mM dithiothreiotol (Sigma), followed by incubation for 2 h at 37°C. The virus was adhered to carbon filmed grids by placing the grid, face down, on droplets of virus suspensions on parafilm. All incubations and washings were then performed with the grids carrying the virus face down on the appropriate droplets at room temperature, starting by blocking with 1% BSA. Incubation with the affinity purified ascites MAb against ISA virus (diluted to 3 µg ml^{-1}) was carried out overnight, at 4°C. After several washings, the specimens were labelled with goat anti-mouse IgG conjugated to 5 nm colloidal gold particles (GAM IgG-5 nm) (BioCell Research Laboratories, Cardiff, UK) for 90 min at room temperature, then washed and finally, negatively stained for 10 s in 0.5% phosphotungstic acid (PTA), pH 7.0. Negative controls were carried out using an irrelevant IgG_1 MAb or buffer replacing the anti-ISA virus MAb. One per cent ammonium acetate with 0.5% BSA was used for all washings and dilutions. Electron microscopy was carried out using a JEOL 1010 electron microscope operated at 100 keV.

Cross-reactivity of anti-ISA virus MAb. Cross-reactivity with other ISA virus isolates was assessed by IF on acetone-fixed infected cell cultures in 96-well culture plates as described below. The IF test was also used for testing of cross-reactivity with other fish viruses occurring in salmonids. Infectious pancreatic necrosis (IPN) virus (a Norwegian SP isolate) and infectious haematopoietic necrosis (IHN) virus (a French isolate) were propagated in SHK-1 cells. Viral haemorrhagic septicaemia (VHS) virus (a Norwegian isolate) does not grow in SHK-1 cells and was propagated in BF-2 cells.

IIF staining of tissue sections. Tissue samples from kidney, liver and heart were collected from moribund fish during a natural ISA outbreak and from fish from the southern coast of Norway where ISA has never been observed. The tissues were frozen in isopentane, chilled in liquid nitrogen, and stored at –80°C. Sections were cut on a cryostat, placed on poly-L-lysine coated slides and fixed in chilled 100% acetone for 10 min. IIF staining was performed as described above for the cultured cells using anti-ISA virus MAb supernatant.

Infectivity titration of ISA virus. Virus infectivity was determined by end-point titration in 96-well culture plates using IF. Fifty µl of serially diluted virus in serum-free culture medium was added to 1 to 2 d old cell cultures containing 150 µl $well^{-1}$ culture medium. Following incubation at 15°C for 6 to 8 d, the culture plates were washed once in PBS, fixed in 80% acetone in distilled water for 20 min and air-dried. The cells were then incubated with 50 µl $well^{-1}$ of FITC-conjugated anti-ISA virus MAb, 1/1000 in PBS with 1% non-fat dry milk at room temperature. After 3 washes in PBS-T, the cells were observed in an inverted fluorescence microscope. To facilitate reading, nuclei were stained by addition of 10 µg ml^{-1} propidium iodide (Sigma) in the second wash. Wells containing at least 1 infected cell were considered to be ISA virus positive, and the $TCID_{50}$ was estimated by the method of Kärber (Kärber 1931).

RESULTS

Production of hybridomas

Six hybridomas produced antibody that reacted only against ISA virus antigen in both ELISA and IIF and were selected for subcloning. Only one of these hybridomas, designated 3H6F8, was stabilized and this clone was further cloned twice and used in the subsequent work. The isotype of this clone was determined to be IgG_1.

Immunofluorescent staining of ISA virus infected SHK-1 cells

Immunofluorescent examination of infected cells showed strong fluorescence in infected cells with no background staining (Fig. 1). Titration of MAb supernatant showed that the supernatant could be diluted 1:1000 without any significant loss in fluorescence intensity.

Fluorescent staining was first detected 24 h postinfection and either appeared as a weak, diffuse staining in the cytoplasm or as a distinct perinuclear staining (Fig. 1a). As the infection proceeded, the fluorescence generally became more intense and several patterns of fluorescence were observed. Some cells showed a dense cytoplasmic staining while others exhibited a more granular type of staining, often concentrated in inclusion-like structures (Fig. 1b, c). In cell cultures that had been infected with a lower infectious dose (approximately 10^{-3} $TCID_{50}$ per cell), positive cells were observed in clusters surrounded by large areas of cells showing no reaction (Fig. 1d). Staining of nuclei was never observed using this antibody and no staining was observed in non-infected cells.

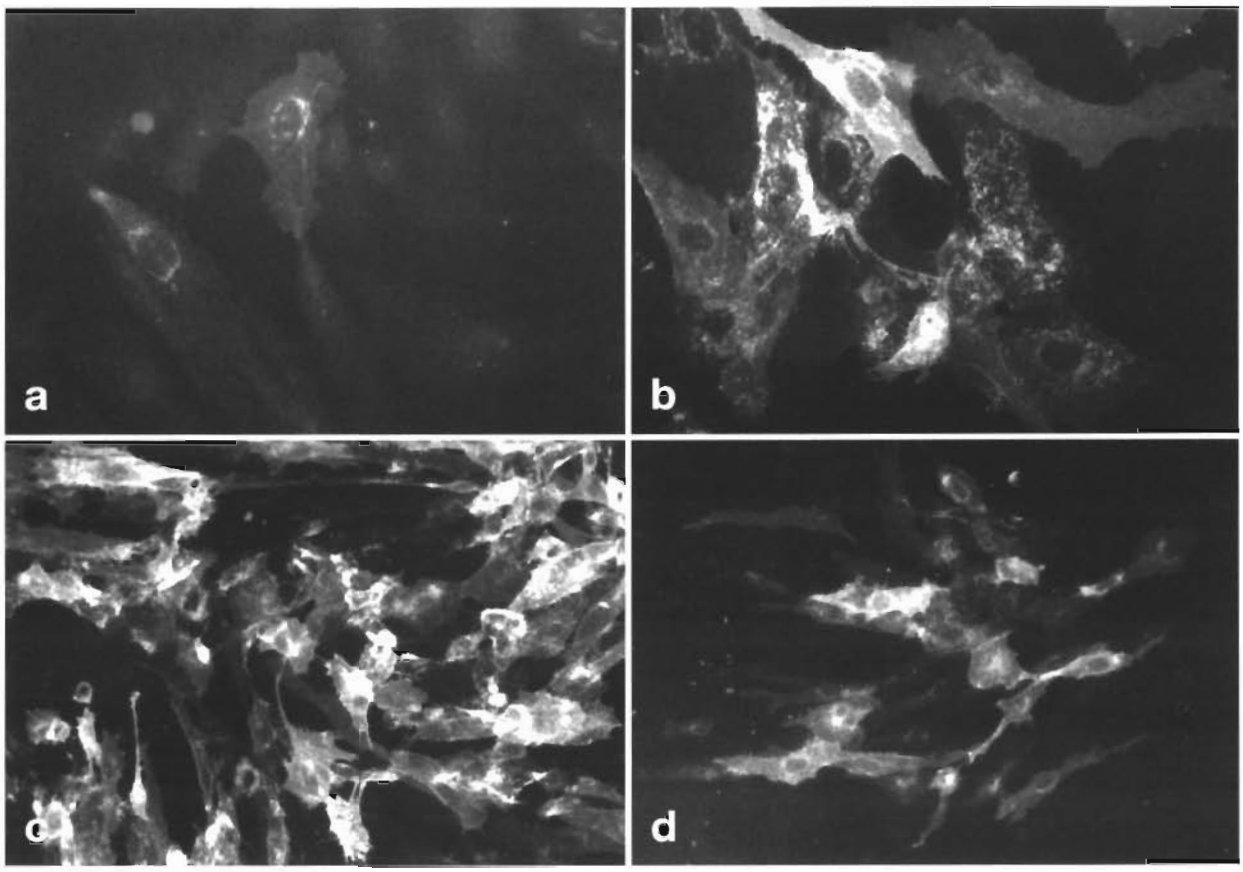

Fig. 1 IIF staining of ISA virus infected SHK-1 cells in 96-well culture plates using the FITC-conjugated anti-ISA virus MAb. (a) Perinuclear staining 24 h postinfection, ×260; (b) diffuse and granular patterns of cytoplasmic staining 4 d postinfection, ×260; (c) patterns of staining 7 d postinfection, ×130; (d) cluster of stained cells in a culture infected at a MOI of 10^{-3} 4 d postinfection, ×130

Virus neutralization and haemagglutination inhibition

It was not possible to determine an exact endpoint for virus neutralization using the immunofluorescence test. However, more than 99% reduction of infected cells was observed in dilutions up to 1/500 000 with MAb ascites while complete neutralization was observed in dilutions up to 1/400. Similarly, a haemagglutination inhibition titre of 1/16 was obtained using MAb supernatant and 1/2000 using ascites.

Immunoelectron microscopy

Immunogold labelling of a purified viral preparation with the anti-ISA virus MAb showed strong surface labelling of the virions (Fig. 2a). No labelling was detected in control preparations using an irrelevant MAb (Fig. 2b) or in preparations of virions treated with bromelain to remove surface spikes (Fig. 2c).

Cross-reactivity of anti-ISA virus MAb

Twelve new strains of ISA virus were obtained (Table 1) from frozen material during this study representing both different geographic locations and points of time. When these strains were tested by IF with the anti-ISA virus MAb, all showed positive reactions with characteristic staining patterns. Furthermore, no specific fluorescence was observed when IPN virus, VHS virus and IHN virus infected cultures were tested by IF using the anti-ISA virus MAb.

Immunofluorescent staining of tissue sections

IIF staining using the anti-ISA virus MAb on frozen tissue sections from moribund Atlantic salmon collected during a natural ISA outbreak revealed strong fluorescence in kidney, heart and liver (Fig. 3). In the kidney, fluorescence was localized both in glomeruli and haematopoietic tissue (Fig. 3a). In the heart, the

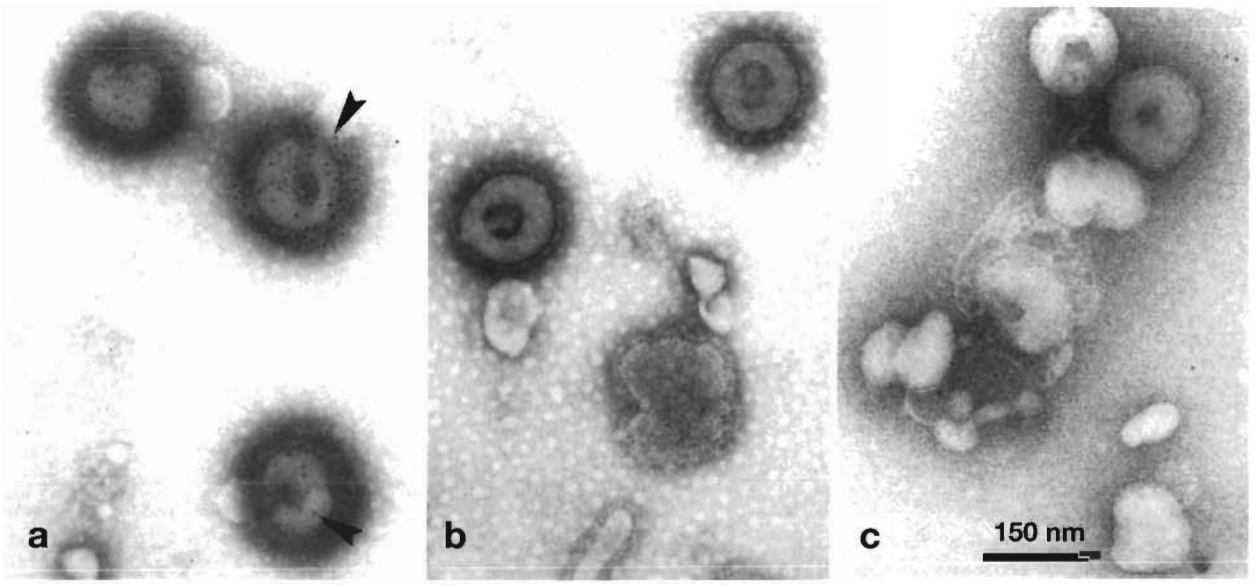

Fig. 2. Transmission electron micrographs of CsCl gradient-purified ISA virus immunogold labelled with goat anti-mouse IgG-5 nm using (a) anti-ISA virus MAb; (b) an irrelevant MAb; (c) anti-ISA virus MAb on bromelain treated virus. ×100 000. Arrows indicate immunogold-labelled MAb attached to virus in (a)

fluorescence was mainly associated with the endocardium (Fig. 3b) and in the liver the fluorescence appeared to be associated with sinusoidal structures (Fig. 3c). No immunostaining was observed using the irrelevant MAb or in tissue sections from the negative control fish.

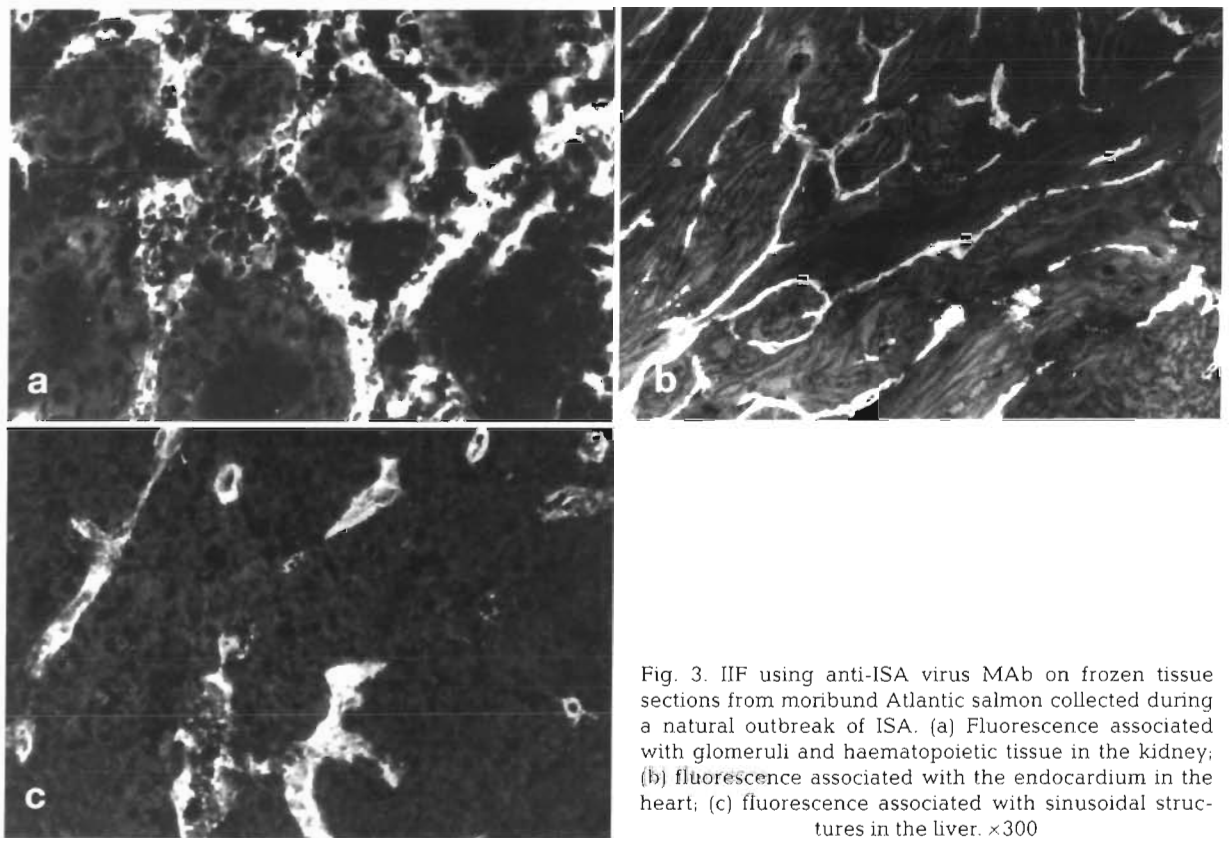

Fig. 3. IIF using anti-ISA virus MAb on frozen tissue sections from moribund Atlantic salmon collected during a natural outbreak of ISA. (a) Fluorescence associated with glomeruli and haematopoietic tissue in the kidney; (b) fluorescence associated with the endocardium in the heart; (c) fluorescence associated with sinusoidal structures in the liver. ×300

Infectivity titrations of ISA virus by immunofluorescence

To evaluate the infectivity titration by IF, parallel serial dilutions of virus were made in eight 96-well culture plates. The cells were evaluated for CPE and fixed in acetone 4 h, 1, 2, 3, 6, 8, 10 and 13 d postinfection. Plates were then stained by IF and infectivity titres calculated.

Results of the comparison of IF and CPE evaluations for estimating virus infectivity are presented in Table 2. The first weak signs of CPE appeared 3 d postinfection. However, the cultures had to be incubated for 2 wk before all infected wells showed CPE. In contrast, positive fluorescence was detected in most of the infected wells following 1 d of incubation. All infected wells were detected after 3 to 4 d of incubation. The intensity of the fluorescent reaction and the number of fluorescing cells increased during the incubation period and the reaction was most easily read following 5 to 7 d of incubation.

Furthermore, to evaluate reproducibility of the IF assay, virus infectivity of 1 aliquot of virus was determined 9 times during the same day using the cell cultures from a similar batch of cells. The results revealed a 3.2-fold range in $TCID_{50}$ titres between the 9 titrations, ranging from 3.2×10^5 to 1.0×10^6 $TCID_{50}$ ml^{-1} Similarly, aliquots of another virus preparation were assayed 6 times on different days during a 4 mo period revealing a 2.5-fold range in $TCID_{50}$ titres, ranging from 1.3×10^5 to 3.2×10^5 $TCID_{50}$ ml^{-1}. No decrease in virus titres was observed during this period.

DISCUSSION

Monoclonal antibodies are useful tools in the diagnosis and control of fish diseases. The development of MAbs against fish viruses infecting salmonids, including IHN virus (Schultz et al. 1985, Winton et al. 1988), IPN virus (Caswell-Reno et al. 1986, Wolski et al. 1986) and VHS virus (Lorenzen et al. 1988), has already been reported. In the present study, we describe the production and characterization of the first MAb against the ISA virus, an orthomyxovirus-like virus pathogenic to Atlantic salmon.

Table 2. Comparison of ISA virus infectivity titre in a tissue culture supernatant evaluated by IF assay and by CPE. Infectivity titres are expressed as $TCID_{50}$ ml^{-1}

Days of incubation	Virus infectivity titres ($TCID_{50}$ ml^{-1}) CPE	IF
0	0	0
1	0	2.0×10^4
2	0	7.9×10^4
3	5.0×10^1	2.0×10^5
6	1.3×10^3	1.6×10^5
8	1.3×10^4	2.5×10^5
10	7.9×10^4	3.2×10^5
13	2.5×10^5	2.5×10^5

The anti-ISA virus MAb showed a strong reaction both with IIF on ISA virus-infected cell cultures, and with ELISA using lysed-infected cell cultures as antigen. ISA virus negative control preparations showed virtually no background reaction. Furthermore, no staining was detected in cell cultures infected with IPN virus, VHS virus and IHN virus. The specificity of the anti-ISA virus MAb was confirmed by immunoelectron microscopy which demonstrated a strong labelling of the surface of purified virions. No labelling was detected in control preparations including preparations where virion spikes had been removed by bromelain treatment.

In general, the MAb showed strong reactivity when used for IIF staining of infected cell cultures, even when the hybridoma supernatant was diluted more than 1/1000. However, the MAb failed to react with viral polypeptides in western blots, under both reducing and non-reducing conditions and no reaction was seen using this antibody on formalin-fixed paraffin-embedded tissue sections from diseased fish (data not shown). These findings suggest that the antigenic determinant recognized by the MAb is a conformationally dependent epitope.

The ISA virus has recently been shown to possess orthomyxovirus-like properties including haemagglutinating activity and receptor destroying enzyme activity (Falk et al. 1997). It is currently not known whether these activities are associated with a single glycoprotein as in influenza C virus, which has only 1 surface glycoprotein, or with 2 different glycoproteins as in influenza A and B viruses. The anti-ISA virus MAb was shown both to neutralize ISA virus infection in cell culture and to inhibit the haemagglutination reaction. Thus, it is concluded that the MAb is specific for a ISA virus polypeptide, which is most likely the haemagglutinin on the virion surface.

Since antigen variation in influenza viruses involves primarily the surface glycopeptides (Krug 1989), it was of particular interest for the potential use in diagnosis to examine the ability of the anti-ISA virus MAb to recognize different strains of the virus. The MAb displayed good reactivity with a total of 13 ISA virus strains examined in the present study. The ability of the MAb to recognize these 13 strains of virus, which were collected from diverse geographic regions over a period from 1989 until the present, indicates that this MAb should be a useful reagent for the development

of diagnostic tests. However, considering the narrow specificity of MAbs in general, care will need to be taken when interpreting results from negative diagnostic tests, particularly as the present MAb appears to bind to a surface polypeptide that is known to show antigen variation.

The slow and often weakly cytopathic growth of ISA virus in cell culture has hampered the study of this virus in tissue cultures and made quantification of the virus in 96-well tissue culture plates difficult. Viruses, including orthomyxoviruses, which often do not induce distinct CPE in cell cultures, have been detected and quantified by different immunological methods or by haemagglutination. In this paper we describe an immunofluorescence-based assay that is simple and rapid, yet sufficiently accurate to provide reliable measurements of titres for ISA virus in cell cultures. Immunofluorescent staining of virus-infected cell cultures is usually performed on cover glass cultures or on chamber slides which is a laborious and time-consuming procedure allowing only a limited number of cultures to be stained simultaneously. We found that an adequate fluorescence signal was obtained in 96-well culture plates despite the loss of some light intensity due to the relatively thick (1 mm) plastic bottoms of the wells. The advantages of this method are the ability to examine many samples and culture plates within a short time period, and the good contrast provided by the immunofluorescence staining which allowed single infected cells to be easily detected. We have also successfully used this system for the detection of other fish viruses including IPN virus, VHS virus and IHN virus grown in various tissue culture systems, although the reaction intensity and readability varied depending on the antibody used (authors' unpubl. data).

The reliability and accuracy of the immunofluorescence test for titration of ISA virus infectivity were determined by comparison with titration by CPE and by repeatedly titrating a virus preparation on the same day and on different days. The results showed a good agreement between immunofluorescent staining and CPE evaluation. However, while the immunofluorescence test could be read following 3 to 4 d of incubation, cultures had to be incubated for 2 wk to achieve the same result by CPE evaluation. Furthermore, there was also a close agreement between the different assays performed on the same and on different days.

Until now, no specific method for detection of the ISA virus in clinical specimens has been available. The diagnosis of ISA has been based on clinical, pathological and haematological findings. However, it has been difficult to diagnose unequivocally early or mild cases of ISA in fish with minor pathological lesions or in fish with multiple diseases. The anti-ISA virus MAb was tested on cryosections from naturally infected Atlantic salmon using IIF. There was considerable reactivity in the liver, kidney and heart of moribund fish with typical ISA symptoms. No immunoreactivity was detected in fish from farms with no history of ISA. Although these results have to be confirmed on a larger series of specimens including fish at different stages of the disease and fish from different natural outbreaks, the anti-ISA MAb shows promise for use in the diagnosis of ISA.

In summary, we have described the development of the first MAb against the ISA virus. This MAb showed good reactivity with various strains of ISA virus from diverse geographic regions collected from 1989 until the present and thus would appear to be an appropriate reagent for the development of diagnostic methods. Using this MAb, a rapid IF assay for detection and titration of ISA virus in cell cultures was developed. In addition, the results using an IIF test on cryosections were also promising for the detection of ISA virus in clinical specimens.

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