Antibody-mediated growth of infectious salmon anaemia virus in macrophage-like fish cell lines

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Infectious salmon anaemia virus (ISAV), a pathogen in marine aquaculture, belongs to the genus Isavirus, family Orthomyxoviridae. There is limited information on how ISAV interacts with host defences. To study ISAV–antibody interactions, virus neutralization (VN) assays were performed in the cell lines CHSE-214, SHK-1 and TO using three strains of ISAV and rabbit or fish anti-ISAV sera. Homologous VN titres of >1:1280 in CHSE-214 cells corresponded to titres of only 1:80 in the macrophage-like fish cell lines SHK-1 and TO, despite using 1000 and 2000 times less virus, respectively. However, rabbit antiserum to infectious pancreatic necrosis virus (IPNV) had a VN titre of 1:10 260 against IPNV in both CHSE-214 and TO cells. Poor ISAV neutralization in TO cells was attributed to Fc receptors mediating virus infectivity, because (1) neutralization by rabbit antiserum to ISAV was increased 48-fold in the presence of staphylococcal Protein A and (2) when using FITC-labelled virus and spectrofluorometry, a significant increase in the intensity of fluorescence of intracellular virus was observed in assays of virus–antiserum mixtures in the absence of Protein A as compared to those in the presence of Protein A. Neutralization of ISAV with fish antisera was observed only in CHSE-214 cells, as Protein A could not restore neutralization in TO cells. These findings demonstrate for the first time antibody-mediated infection of macrophage-like fish cell lines by a fish virus, ISAV, and, as ISAV in Atlantic salmon targets leukocytic and endothelial cells, this may have implications for ISA pathogenesis and vaccination.

INTRODUCTION

Infectious salmon anaemia virus (ISAV) is the cause of infectious salmon anaemia (ISA), which is characterized by severe anaemia and high mortality in farmed Atlantic salmon. The pathological changes of the disease include exophthalmia, leukopenia, ascites, congestion and enlargement of liver and spleen, congestion in the intestinal mucosa, haemorrhagic liver necrosis and petechiae in the visceral fat. The clinical disease was first recognized in Norway (Thorud & Djupvik, 1988) and has since been reported in Canada (Mullins et al., 1998), Scotland (Rodger & Richards, 1998), the Faro Islands (Anon., 2000) and Maine, USA (Bouchard et al., 2001). The virus has also been detected in diseased, farmed Coho salmon in Chile (Kibenge et al., 2001a) and in apparently normal wild fish (sea trout, Atlantic salmon and brown trout) from Scotland (Raynard et al., 2001). Sea trout, brown trout and rainbow trout have been shown experimentally to be asymptomatic carriers of the virus (Nylund & Jakobsen, 1995; Nylund et al., 1995, 1997). The immune response in Atlantic salmon does not provide full protection against ISAV infection (Falk et al., 1995) and farmed Atlantic salmon that recover from clinical ISA continue to shed the virus for long periods of time, but detection of ISAV in carrier fish required the use of RT-PCR (Devold et al., 2000), which suggests that viral RNA is not abundant. However, such fish can transmit ISA to healthy Atlantic salmon upon co-habitation (Nylund et al., 1997).

Morphological, biochemical and replication properties of ISAV place the virus in the family Orthomyxoviridae (Falk et al., 1997; Kim & Leong, 1999). Similar to influenza viruses, the ssRNA genome of ISAV comprises eight segments of negative polarity. Comparison of ISAV proteins with those of other orthomyxoviruses revealed low amino acid identity of between <13 and <25 % (Krossøy et al., 2001; Kibenge et al., 2001b; Ritchie et al., 2001), supporting the proposal to assign ISAV to a new, fifth genus within the Orthomyxoviridae, genus Isavirus (Anon., 2001).

The mechanisms of virus persistence and pathogenesis in ISAV infection at the cellular level are not well studied. The phenomenon of persistent ISAV infection in fish is unusual for orthomyxoviruses, as there is no evidence of persistence of influenza virus genetic material in any animal species. Influenza viruses are cleared from ducks in approximately 7 days (Webster, 1999), although they can persist in carrier...
cultures (Urabe et al., 1993). In Atlantic salmon, ISAV seems to target leukocytic (Falk et al., 1995), endothelial (Falk & Dannevig, 1995) and endothelial-associated (Falk et al., 2001) cells, while in vitro, it readily replicates with production of cytopathic effects (CPE) in salmon head kidney (SHK-1) (Dannevig et al., 1995), TO (Wergeland & Jakobsen, 2001) and Atlantic salmon kidney (ASK-2) (Rolland et al., 2002) cells, which are macrophage-like cell lines (Dannevig et al., 1997; Wergeland & Jakobsen, 2001; Rolland et al., 2002). Some strains of ISAV can also replicate and cause CPE in the Chinook salmon embryo (CHSE-214) cell line (Bouchard et al., 1999; Kibenge et al., 2000; Griffiths et al., 2001). Virus replication may also occur in Atlantic salmon (AS) (Sanchez et al., 1993; Sommer & Mennen, 1997) and rainbow trout gills (RTgill-W1) (Bols et al., 1994) cell lines, but, in these cases, the virus is non-cytopathic.

It is known that macrophages of mammals and birds contain Fc receptors, which allow them to internalize and digest virus particles coated with antibody (Mantovani et al., 1972). Antibody-enhanced infection occurs when monocytes and macrophages are more efficiently infected by virus–antibody complexes via Fc receptor-mediated endocytosis than virus alone (Porterfield, 1986). A number of human and animal viruses belonging to at least 11 different virus families have been shown to be capable of utilizing this mechanism of infection. These include Dengue virus and related flaviviruses (Halstead et al., 1984; Peiris et al., 1981), respiratory syncytial virus (Krilov et al., 1989), rabies virus (King et al., 1984), human immunodeficiency virus (Homsy et al., 1989; Takeda et al., 1988), influenza A virus (Ochiai et al., 1988), feline infectious peritonitis virus (FIPV) (Stoddart, 1989) and, most recently, coxsackievirus B3 (Girn et al., 2002). Many of these viruses demonstrating antibody-dependent enhancement in vitro have been associated also with higher morbidity or mortality in cases of prior immunity (Sullivan, 2001). However, in the case of influenza virus, antibody-mediated internalization and growth of influenza A virus NWS (H1N1) has been demonstrated only in the cultured murine macrophage-like cell line P388D1 in the presence of subneutralizing antiviral IgG (Ochiai et al., 1988). Although Fc receptors for fish IgM have been demonstrated on fish leukocytes (O’Dowd et al., 1998; Haynes et al., 1988), there is no report to date of Fc receptor-mediated infection of macrophages by fish viruses. In this study, we investigated whether antiviral antibodies enable the fish orthomyxovirus ISAV to infect the macrophage-like fish cell lines SHK-1 and TO, thereby abrogating virus neutralization (VN) in vitro.

METHODS

Viruses and cell cultures. Three different strains of ISAV, NBISA01, RPC-980-049(1) and RPC-990-002(4), were propagated in CHSE-214, SHK-1 and TO cell lines, as described previously (Kibenge et al., 2000, 2001a, b). Virus titration was performed on 24-h-old cell monolayers in 48-well plates and the virus titre in each cell line was determined from endpoint CPE using the procedure described by Reed & Muench (1938). Virus titres are expressed as TCID50 per 100 μl.

Polyclonal antibody preparations. The preparation of rabbit polyclonal antisera to purified ISAV isolates RPC-980-049(1) and RPC-990-002(4) has been described previously (Kibenge et al., 2000). Atlantic salmon sera consisted of a pooled field sample collected from farmed fish in New Brunswick, Canada (Kibenge et al., 2002), and pooled serum collected from Atlantic salmon experimentally infected with ISAV strain NBISA01. Rainbow trout serum was also collected from fish experimentally infected with ISAV strain NBISA01. For production of fish anti-ISAV sera, Atlantic salmon or rainbow trout were inoculated intraperitoneally with 105 TCID50 ISAV strain NBISA01 0.2 ml−1 per fish and kept in a fresh water tank at 10°C for at least 84 days. Normal rabbit serum and sera from uninfected control rainbow trout and Atlantic salmon served as negative control sera. Polyclonal rabbit anti-IPNV serum to infectious pancreatic necrosis virus (IPNV) strain FVX-8 used for VN assays of IPNV was provided by Dr C. Yason (Atlantic Veterinary College Virology Diagnostic Laboratory). All serum samples were heat-inactivated at 56°C for 30 min prior to use in order to destroy complement activity. This treatment also served to inactivate any ISAV (Falk et al., 1997) that might have contaminated the fish sera used.

VN assays. VN tests were carried out on 24-h-old cell monolayers in 48-well cell culture plates as described previously (Kibenge et al., 2001b). Cultures were examined microscopically for CPE to determine VN test results after 10 days of incubation (14 days in the case of the CHSE-214 cell line) at 16°C.

Blocking of Fc receptors. Staphylococcal Protein A (Sigma) was resuspended to a concentration of 1 mg ml−1 in sterile distilled deionized water. Parallel VN assays were conducted in TO cell monolayers using ISAV strain NBISA01 and rabbit or Atlantic salmon anti-ISAV sera in the presence or absence of Protein A, as described previously (Olsen et al., 1992), with slight modifications. Briefly, Protein A was added to a final concentration of 200 μg ml−1 to the pre-incubated virus–antiserum mixtures, which were incubated for a further 1 h at room temperature. Virus–antiserum mixtures were made in media without FBS in order to avoid competition for Protein A binding. TO cell monolayers in 48-well cell culture plates were inoculated with the virus–antibody–Protein A mixtures and incubated for an additional 1 h at room temperature. Residual virus–antibody complexes were then removed and fresh maintenance medium was added. Cultures were then incubated and monitored as described for the VN assays. Cells infected with virus in the presence or absence of Protein A served as controls.

Preparation of FITC-labelled ISAV. ISAV strain RPC-980-280(2) was propagated in TO cells. Infected cell culture lysates were clarified at 3000 g for 30 min in a JA14 Beckman rotor. The cell pellet was saved and suspended in 1× TNE (10 mM Tris/HCl, 0-1 M NaCl and 1 mM EDTA, pH 7-9). Virus was purified on a Ficoll-400 (Amersham Pharmacia) step gradient and a sucrose cushion as described previously (Kibenge et al., 2000). Purified virus (1200 μl) with a concentration of 0.5 mg viral protein ml−1 was reacted with an equal volume of 0.1 mg FITC ml−1 dissolved in 0.5 M bicarbonate buffer (pH 9-5) for 1 h at room temperature, as described previously (Nichols et al., 1993). Unconjugated dye was removed by passing the virus preparation through a Bio-Gel P-6DG column (Bio-Rad). Labelled virus was eluted using an equal volume of 1× PBS and was then passed through a 0.45 μm syringe filter to eliminate virus aggregates. It was then stored at 4°C. Labelled virus was divided into six equal volumes of 350 μl and each volume was...
Demonstration of antibody-mediated virus uptake using FITC-labelled ISAV. TO cells (1×10^4 ml^-1) were grown in slide flasks (Fisher) (3 ml per flask) or 6-well tissue culture plates (Costar) (3 ml per well) overnight. Parallel VN assays were then set up using FITC-labelled ISAV and rabbit anti-ISAV serum in the presence and absence of staphylococcal Protein A. For this, 350 μl FITC-labelled ISAV was incubated with equal amounts of 1:640 dilutions of antiserum for 1 h at room temperature. Preliminary experiments established that Protein A, when used at a concentration of 200 μg ml^-1, could completely block the enhancing property of the rabbit anti-ISAV serum at a dilution of 1:640 in TO cells. Protein A was, therefore, added to the virus-antibody mixture to a final concentration of 200 μg ml^-1 and incubated for 1 h at room temperature. Cell monolayers were then inoculated with the FITC-labelled ISAV antiserum–protein A mixture and incubated for 1 h at room temperature. TO cells inoculated with FITC-labelled ISAV alone were used as the positive control, whereas uninfected TO cells and TO cells inoculated with FITC were used as the negative controls. All flasks and plates were incubated at 16°C for an additional 3 h and were then analysed using fluorescent microscopy or spectrofluorometry.

Slide flasks were processed for fluorescent microscopy by removing the inoculum from the flasks and washing the cell monolayers three times with 1× PBS. Slides were then detached from the flasks, fixed with 99% ethanol for 10 min at 4°C and air dried. Slides were analysed using a Fluoview 300 confocal laser scanning microscope (Olympus America) at a magnification of ×400 and Fluoview software, version 3.0.

The 6-well tissue culture plates were processed for spectrofluorometry by removing the inoculum from the tissue culture plate and washing the cell monolayers with 1× PBS. Cells were detached using trypsin and resuspended in 1 ml growth medium containing 10% FBS. Cells were pelleted at 500 g for 10 min and resuspended in 1× PBS. A 50 μl cell suspension from each sample was placed in a single well of a Nunclon F microwell plate and used to measure the intensity of fluorescence using a SpectraMAX Gemini XS spectrofluorometer (Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Each sample was tested in five replicates. Results were analysed by one-way ANOVA.

RESULTS

Comparison of ISAV VN in CHSE-214 and macrophage-like cells

In preliminary experiments of VN assays with rabbit antisera to ISAV using the different fish cell lines, it was observed consistently that VN antibody titres in the SHK-1 cell line were very low and did not accurately reflect the antigenic relationships between different ISAV strains. VN by homologous antiserum could be demonstrated in CHSE-214 cells, while the same virus-antibody mixtures showed little or no neutralization when tested in SHK-1 cells. Inoculation of SHK-1 cell monolayers with the virus–antibody mixtures resulted in CPE. Thus, meaningful VN data could not be obtained with the SHK-1 cell line. Therefore, it was decided to investigate whether macrophage-like fish cell lines are infected by antibody-bound ISAV. Thus, VN assays were set up with both rabbit and fish polyclonal anti-ISAV antibodies using the same virus strains on three permissive fish cell lines as indicator systems.

VN antibody titres obtained against three different ISAV strains on the three cell lines (CHSE-214, SHK-1 and TO) with four different ISAV antisera are summarized in Table 1. Rabbit and Atlantic salmon antisera showed higher VN antibody titres in the CHSE-214 cell line than in the SHK-1 and TO cell lines for the three different strains of ISAV used. In the most extreme case, rabbit anti-ISAV serum to ISAV strain RPC-990-002(4) had a homologous VN antibody titre of 1:4800 in CHSE-214 cells but only

Table 1. VN titres using rabbit and fish anti-ISAV sera on three different fish cell lines

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>CHSE-214 cell line</th>
<th>SHK-1 cell line</th>
<th>TO cell line</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Anti-ISAV serum*</td>
<td>Anti-ISAV serum*</td>
<td>Anti-ISAV serum*</td>
</tr>
<tr>
<td></td>
<td>RPC-980-049(1)</td>
<td>RPC-980-049(1)</td>
<td>RPC-980-049(1)</td>
</tr>
<tr>
<td>RPC-990-002(4)</td>
<td>960†</td>
<td>2560</td>
<td>80</td>
</tr>
<tr>
<td>RPC-990-002(4)</td>
<td>640</td>
<td>4800</td>
<td>80</td>
</tr>
<tr>
<td>RPC-980-049(1)</td>
<td>&gt; 1280</td>
<td>480</td>
<td>40</td>
</tr>
<tr>
<td>NBISA01</td>
<td>30</td>
<td>120</td>
<td>30</td>
</tr>
<tr>
<td>RPC-990-002(4)</td>
<td>80</td>
<td>20</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>RPC-990-002(4)</td>
<td>40</td>
<td>20</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>RPC-980-049(1)</td>
<td>40</td>
<td>20</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

*All anti-ISAV sera were raised in the laboratory to different strains of ISAV, except for the Atlantic salmon serum, which was a pool of field serum samples from a natural ISA outbreak in Atlantic salmon. Animal species are shown in parentheses.

†VN titres are expressed as the highest dilution of serum to completely neutralize 100 TCID50 of the respective ISAV strain.
Titration of 100 TCID$_{50}$ ISAV strain NBISA01 used in VN assays in three different fish cell lines

Virus titres were measured by endpoint CPE and are expressed as log$_{10}$ TCID$_{50}$ 100 µl$^{-1}$.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Titration on cell line</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CHSE-214</td>
</tr>
<tr>
<td>CHSE-214</td>
<td>2-0</td>
</tr>
<tr>
<td>SHK-1</td>
<td>&lt;2-0</td>
</tr>
<tr>
<td>TO</td>
<td>&lt;2-0</td>
</tr>
</tbody>
</table>

1:40 in SHK-1 cells and <1:10 in TO cells. With the Atlantic salmon and rainbow trout anti-ISAV sera, VN was observed only in CHSE-214 cells.

Titration of 100 TCID$_{50}$ 100 µl$^{-1}$ of virus in different fish cell lines

The amount of virus used in the VN assay (100 TCID$_{50}$ 100 µl$^{-1}$) for each cell line was titrated on all three cell lines to rule out excessive amounts of virus as a cause of poor ISAV neutralization on SHK-1 and TO cells. Table 2 shows the results of these titrations. It is apparent that the 100 TCID$_{50}$ of the CHSE-214 cell system was 1000 and 2000 times more than that of the SHK-1 and TO cell systems, respectively. This is in contrast to the fact that the CHSE-214 cell system showed the highest VN antibody titres against ISAV. This indicated that the true VN antibody titres of the rabbit and fish anti-ISAV sera used were higher than those detected with the CHSE-214 cell system. We considered this to be suggestive of antibody-mediated growth of ISAV in macrophage-like fish cell lines SHK-1 and TO. During the course of this work, it was determined that the SHK-1 cell line at higher passages became less sensitive to ISAV. Since the pattern of neutralization of ISAV was the same for fish and rabbit antisera in both TO and SHK-1 cell lines (Table 1), it was decided to use only TO and CHSE-214 cells for further investigations.

IPNV VN in TO cells

In order to determine whether effective VN is possible in TO cells, VN of IPNV in TO cells was compared to that in CHSE-214 cells using rabbit anti-IPNV serum. IPNV was neutralized to the same degree (VN titre of 1:10 260) in both CHSE-214 and TO cell lines. This demonstrated that the poor neutralization of ISAV in TO cells was unique to ISAV.

ISAV VN in TO cells increases significantly in the presence of staphylococcal Protein A

To test the hypothesis that the poor neutralization of ISAV by anti-ISAV sera on macrophage-like fish cell lines was due to Fc receptor-mediated uptake of antibody-coated virus particles by the cells, staphylococcal Protein A was used to block Fc receptors on the antibody prior to use in VN assays. Appropriate VN controls in the absence of Protein A were also carried out. When Protein A was used in the VN assay with ISAV strain NBISA01 in TO cells, an antibody titre of 1:960 was obtained with rabbit anti-ISAV serum compared to a titre of 1:20 in TO cells without Protein A (Table 3). This was a 48-fold increase in ISAV VN titre in TO cells and a 1:5-fold increase compared to that obtained in CHSE-214 cells (Table 1). Protein A by itself did not adversely affect ISAV infectivity. This finding indicated that ISAV VN in TO cells could be increased significantly by the pre-treatment of the virus–antibody mixture with Protein A. However, Protein A did not affect the VN titre in CHSE-214 cells (Table 3). Because Protein A is known to bind the Fc moiety of immunoglobulins (Olsen et al., 1992; Stoddart, 1989), these data support the conclusion that rabbit immunoglobulins could bind to receptors on TO cells (possibly Fc receptors) and that poor ISAV VN in TO and SHK-1 cells may be due to Fc receptor-mediated uptake and subsequent replication of ISAV in these macrophage-like fish cell lines. There was no VN when the rabbit anti-ISAV serum was used at dilutions of 1:40 to 1:320 in TO cells in the presence of Protein A, indicating that the binding effect of Protein A could be diluted out with higher antibody concentrations. In dilutions of 1:640 to 1:1280, Protein A could completely block antibody-mediated uptake of the virus. A similar biphasic response was also reported for FIPV–antibody–macrophage interactions (Stoddart, 1989). Protein A could not restore ISAV neutralization by fish antisera in TO cells (Table 3), due either to the low affinity of Protein A to fish immunoglobulins or to insufficient amounts of Protein A used in the assay relative to the number of Fc receptors on fish immunoglobulins.

Demonstration of Fc receptor-mediated uptake of ISAV

To demonstrate that ISAV uptake by cells in the presence of anti-ISAV serum was mediated by Fc receptors, the virus...
was purified and labelled with FITC. Intracellular virus was detected by two different methods, confocal laser scanning microscopy and spectrofluorometry. After inoculation, TO cells were incubated with FITC-labelled ISAV for 4 h to allow the entry of the virus into the cells (Eliassen et al., 2000). Confocal laser scanning microscopy showed an apparent increase in the fluorescence of TO cells infected with ISAV in the presence of rabbit anti-ISAV serum (Fig. 1b) as compared to TO cells infected with virus alone (Fig. 1a), i.e. antiviral antibodies facilitated the entry of virus into the cells. The intensity of fluorescence was reduced significantly when the experiment was carried out in the presence of Protein A (Fig. 1c), i.e. antibody-mediated uptake of the virus was blocked by Protein A, thereby neutralizing the virus in macrophage-like fish cells. No fluorescence was observed in uninfected TO cells (Fig. 1d). Analysis of serial optical sections of the TO cell monolayer infected with FITC-labelled ISAV and rabbit anti-ISAV serum (Fig. 1b) with a depth increment of 0.5 µm showed maximum fluorescence between 6 and 9 µm (Fig. 2c, d). Because the thickness of the cell membrane is only 7–10 nm, these data suggested that most of the fluorescence was intracellular, i.e. most of the labelled virus entered the cells in the presence of virus-specific antibodies.

Fluorescence of intracellular virus was detected by spectrofluorometry. Analysis was performed 4 h after the VN assays using FITC-labelled ISAV. For this, all cells were detached from each well of a 6-well plate and resuspended in 1× PBS to measure the intensity of fluorescence. Significant reduction in fluorescence intensity was observed in TO cells infected with FITC-labelled ISAV treated with rabbit anti-ISAV serum and Protein A (Fig. 3, lane S1) as compared to TO cells infected with FITC-labelled ISAV treated with rabbit anti-ISAV serum in the absence of Protein A (Fig. 3, lane S2) (P = 0.018) or TO cells infected with FITC-labelled ISAV alone (Fig. 3, lane S3) (P = 0.0027). This observation demonstrated further that Protein A blocked the Fc receptor-mediated uptake of antibody-coated ISAV and allowed VN by rabbit anti-ISAV serum. The difference

![Fig. 1](http://vir.sgmjournals.org) Detection of intracellular virus by confocal laser scanning microscopy in TO cells infected with FITC-labelled ISAV (magnification, ×400). TO cells infected with (a) FITC-labelled ISAV, (b) FITC-labelled ISAV treated with 1:640 rabbit anti-ISAV serum and (c) FITC-labelled ISAV treated with 1:640 rabbit anti-ISAV serum and staphylococcal Protein A. (d) Uninfected TO cells. An apparent increase in the fluorescence of TO cells infected with ISAV in the presence of rabbit anti-ISAV serum (b) was observed as compared to TO cells infected with virus alone (a), indicating that ISAV was not neutralized by rabbit antiserum due to Fc receptor-mediated uptake of antibody-coated virus. Fluorescence was reduced significantly when the experiment was carried out in the presence of protein A (c) due to the blocking of Fc receptor-mediated uptake of the virus. No fluorescence was observed in uninfected TO cells (d).

![Fig. 2](http://vir.sgmjournals.org) Confocal laser scanning microscopic analysis of serial optical sections of the TO cell monolayer infected with labelled ISAV and rabbit anti-ISAV serum to ISAV strain RPC-990-002(4) (see Fig. 1b) with a depth increment of 0.5 µm (magnification, ×400): (a) 0 µm, (b) 3 µm, (c) 6 µm, (d) 9 µm, (e) 12 µm and (f) 15 µm depth. Maximum fluorescence was observed between 6 and 9 µm (c, d), suggesting that most of the fluorescence was intracellular; the thickness of the cell membrane is 7–10 nm.
observed between the fluorescence intensity of TO cells infected with FITC-labelled ISAV treated with rabbit anti-ISAV serum in the absence of Protein A (Fig. 3, lane S2) and TO cells infected with FITC-labelled ISAV treated with rabbit antiserum; S3, TO cells infected with FITC-labelled ISAV alone. Significant reduction in the intensity of fluorescence was observed in S1 as compared to S2 ($P=0.018$) and S3 ($P=0.0027$). No significant difference ($P=0.8190$) was observed between the fluorescence intensity of S2 and S3.

These observations are consistent with results obtained from VN assays and confocal microscopic analysis and thus show that rabbit antiviral antibodies bind to a receptor (possibly Fc receptor) on TO cells and facilitate ISAV entry into the cell and that this can be blocked by Protein A.

**DISCUSSION**

In this study, we investigated whether antiviral antibodies enable the fish orthomyxovirus ISAV to infect macrophage-like fish cell lines, because VN by homologous antiserum could be demonstrated in CHSE-214 cells, while the same virus–antibody mixtures showed little or no neutralization when tested on SHK-1 and TO cells. Inoculation of SHK-1 and TO cell monolayers with the virus–antibody mixtures resulted in CPE. Thus, meaningful VN data are difficult to obtain on these cell lines. Although Falk *et al.* (1998) reported that a monoclonal antibody (mAb) 3H6F8 against ISAV neutralized virus in SHK-1 cells, this neutralization was based on fluorescent antibody staining of the cells after incubation for 3 days and the exact endpoint for VN could not be determined. Moreover, the ability of a mAb to neutralize virus does not rule out the possibility of polyclonal antiserum containing neutralizing and/or nonneutralizing antibodies that may mediate enhancement of virus infectivity. In the present study, higher ISAV neutralizing antibody titres, as evidenced by complete absence of CPE, were obtained in the CHSE-214 cell system than in the SHK-1 and TO cell systems with the same antiserum. We then used the following three experiments to show that the poor neutralization of ISAV on macrophage-like fish cell lines is due to antibody-mediated growth of the virus: (1) titration of 100 TCID$_{50}$ virus $100 \mu l^{-1}$ in different fish cell lines showed that the CHSE-214 cell system used 1000 and 2000 times more virus than the SHK-1 and TO cell systems, respectively, but showed higher VN titres. This indicated that the true VN antibody titres of the antisera used are higher than the titres obtained in CHSE-214 cells; (2) anti-IPNV serum neutralized IPNV to the same degree in both CHSE-214 and TO cells, indicating that the poor neutralization of ISAV in TO cells was unique to ISAV; and (3) ISAV VN in TO cells was increased significantly in the presence of Protein A, which binds the Fc moiety of immunoglobulins. FITC-labelled ISAV and confocal laser scanning microscopic (Figs 1 and 2) and spectrofluorometric (Figs 3 and 4) analyses were then used to show that antibody-mediated entry of the virus into TO cells may occur via Fc receptors. The two proposed mechanisms by which ISAV might infect fish macrophages, depending on the presence or absence of antiviral antibody, are depicted in Fig. 5.

We used the VN assay previously on ISAV in TO cells with rabbit anti-ISAV sera to study the antigenic relationships of ISAV isolates (Kibenge *et al.*, 2001b). In that study, VN antibody titres obtained were sufficient to group the ISAV isolates into two main haemagglutinin subtypes. TO cells were ideal for that study because all known ISAV isolates
replicate with clearly discernible CPE in this cell line. In the present study, we obtained higher VN antibody titres in the VN assay with the CHSE-214 cell system, whose 100 TCID₅₀ had 2000 times more virus than that of the TO cell system. The CHSE-214 cell line, however, also has the disadvantage of not showing CPE when inoculated with some ISAV isolates (e.g. the ISAV isolates from Norway and Scotland) (Kibenge et al., 2000), and the low virus yields of CPE-positive ISAV isolates in this cell line suggest that it might not be sensitive enough to detect poorly neutralized virus. All three strains of ISAV used in this study [NBISA01, RPC-980-049(1) and RPC-990-002(4)] were cytopathic in the three fish cell lines (CHSE-214, SHK-1 and TO) and, therefore, the VN assays were easy to read microscopically.

Antibody-mediated growth of viruses is a well-described phenomenon in mammals and birds (Sullivan, 2001). However, this is the first report of antibody-mediated infection of fish cells by a fish virus. This phenomenon was demonstrated with rabbit anti-ISAV sera in TO cells, suggesting that this macrophage-like fish cell line may have Fc receptors for mammalian immunoglobulins. The possibility of this is supported by the fact that a mammalian homologue of FcεRI (the Fc receptor for IgE) chain exists in carp (Fujiki et al., 2000). In mammals, the existence of specific Fc receptors for five classes of immunoglobulins (FcαR, FcδR, FcεR, FcγR and FcμR) has been recognized (Gessner et al., 1998). Our study shows that other mammalian Fc receptor homologues may also exist in teleost macrophages. Fc receptors for shark IgM have been demonstrated on shark leukocytes and high levels of immune complex receptors, possibly Fc receptors, have also been demonstrated on Atlantic salmon leukocytes (O’Dowd et al., 1998; Haynes et al., 1988). Although mammalian IgM and complement can also mediate antibody-dependent enhanced virus infectivity via macrophage C3 receptors (Cardosa et al., 1983), that mechanism was considered unlikely in the present study because we used heat-inactivated anti-ISAV sera.

Protein A could not restore the neutralization of ISAV by fish antisera in TO cells (Table 3), due either to the low affinity of Protein A for fish immunoglobulins or to insufficient amounts of Protein A used in the assay relative to the number of Fc molecules on fish immunoglobulins. An alternative hypothesis that could be derived from the data is that only a small proportion of ISAV is able to infect CHSE-214 cells and it is an epitope that permits virus entry into CHSE-214 cells that is primarily seen and neutralized by rabbit antiserum. However, since the pattern of neutralization of ISAV was the same for fish and rabbit antiserum on the three cell lines (Table 1), it is considered that poor neutralization of ISAV by fish antiserum in TO and SHK-1 cells is also Fc-mediated. It is also possible that macrophage-like fish cell lines have different types of receptors that allow binding of fish or mammalian immunoglobulins. Our preliminary observations on the ASK-2 cell line (another macrophage-like fish cell line) indicate that in this cell line, rabbit anti-ISAV serum, but not fish anti-ISAV serum, neutralizes the virus very well (unpublished data). A previous study in fish described antibody-enhanced infectivity of fish rhabdoviruses that did not appear to involve Fc or C3 receptors but an unknown mechanism (Clerx et al., 1978).

Several different approaches have been used in the past to demonstrate conclusively antibody-mediated internalization of viruses in vitro via Fc receptors, including blocking with monoclonal anti-Fc receptor IgG and its Fab fragment (Peiris et al., 1981) with heat-aggregated IgG (Daughaday et al., 1981; Lewis et al., 1988) or by binding with staphylococcal Protein A before inoculation (Chanas et al., 1982). The high affinity of Protein A for the Fc moiety of the antibodies was exploited elegantly by Stoddart (1989) to

![Fig. 5. Proposed mechanisms by which ISAV may infect fish macrophages. (a) Virus attachment followed by virus receptor-mediated endocytosis in all permissive cells (Eliassen et al., 2000). (b) Virus bound by specific antibody followed by Fc receptor-mediated endocytosis in fish macrophages. (c) VN in macrophage-like cell line TO in the presence of staphylococcal Protein A.](http://vir.sgmjournals.org)
demonstrate Fc receptor-mediated uptake of FIPV. In those studies, it was observed that Protein A reduced dramatically the level of FIPV infectivity in the presence of enhancing antibodies. This observation indicated that binding of Protein A to the Fc moiety could block the attachment of antibody-coated virus to Fc receptors and inhibit antibody-enhanced infection of the virus (Olsen et al., 1992). Thus, Protein A can be used to demonstrate Fc-mediated uptake of viruses, as has been done in the present study.

Since ISAV in Atlantic salmon seems to target leukocytic, endothelial and endothelial-associated cells, it seems reasonable to speculate that Fc-mediated antibody-dependent enhancement of ISAV infection may occur in vivo. This would accelerate the disease process by efficiently and specifically delivering virus to the very target cells within which the virus replicates. Additionally, such an infection, if non-cytolytic, would facilitate virus persistence in fish infected with ISAV. Conceptually, such infection-enhancing antibodies may be more effective when neutralizing antibodies are either absent, such as following antigenic drift and shift of the virus, or consumed by virus excess.

In conclusion, our study has demonstrated for the first time antibody-mediated uptake and replication of a fish virus, ISAV, in macrophage-like fish cell lines SHK-1 and TO. Blockage of this mode of virus infection using Protein A suggests that this is an Fc-mediated infection of cells. Experiments with purified immunoglobulin Fc and Fab fragments need to be done to prove definitively that the antibody-mediated uptake of ISAV in fish macrophage-like cells demonstrated here is mediated by Fc receptors. Systematic analysis of ISAV proteins using both neutralizing and non-neutralizing mAbs is also necessary to identify the ISAV epitopes responsible for this phenomenon. Similarly, studies are necessary to test whether antibodies to ISAV could cause disease enhancement or not. Findings from these studies will bear directly on current efforts to develop ISAV vaccines that have substantial preventative value.

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