Multiplication and haemadsorbing activity of infectious salmon anaemia virus in the established Atlantic salmon cell line

Ann-Inger Sommer and Saskia Mennen
Norwegian Institute of Fisheries and Aquaculture Ltd, N-9005 Tromsø, Norway

Infectious salmon anaemia virus (ISAV), which previously had never been isolated in any of the commercially available established fish cell lines, was successfully propagated in the continuous cell line Atlantic salmon (AS). The yield of infectious ISAV increased with the incubation time of virus-inoculated cells, demonstrated by in vivo infectivity trials in groups of Atlantic salmon. Trypsin treatment of the virus was not necessary for primary infection of AS cells with salmon-grown ISAV. The infection was non-cytopathic, but it was possible to detect virus-infected cells by a haemadsorption centre assay using Atlantic salmon erythrocytes. Pleomorphic enveloped virus particles were seen by transmission electron microscopy of infected AS cells. Elongated forms were observed, but spherical particles with diameters of 90–130 nm were commonest. Growth of ISAV was inhibited by actinomycin D but not by 5-bromo-2-deoxyuridine treatment, which indicates that ISAV may be an aquatic orthomyxovirus.

Infectious salmon anaemia (ISA) is a disease of farmed Atlantic salmon (Salmo salar L.) in Norway. The first outbreak of ISA was recorded at the end of 1984 and reached a peak incidence in 1991. The disease is characterized by severe anaemia in the terminal stages and mortality is usually high (Thorud & Djuvpik, 1988), but asymptomatic ISA virus (ISAV) infections have been demonstrated in other salmonids (Nylund et al., 1994). Enveloped 100 nm virus particles have been detected in tissue from ISA-diseased Atlantic salmon by transmission electron microscopy (TEM) studies (Nylund et al., 1995). ISAV has not previously been isolated in any of the commercially available continuous fish cell lines, but the establishment of a long-term cell line from Atlantic salmon head kidney which supports ISAV replication has been reported (Dannevig et al., 1995). ISAV has also been isolated in cultures of head kidney macrophages. Virus infection in these primary cell cultures was non-cytopathic and was demonstrated by means of in vivo infectivity studies in salmon and by electron microscopy, since no reliable immunoassay was available (Sommer & Mennen, 1996). Previous studies of cytopathic changes after inoculating 34 different established fish cell lines with ISAV were negative (M. Yoshimizu, personal communication). In two of the cell lines, AS (Atlantic salmon) and CHSE-214 (Chinook salmon embryo) cells, ISAV was passaged eight times without observation of any cytopathic effects (CPE) (A.-I. Sommer, unpublished results).

In this study, we investigated the possible non-cytopathic propagation of ISAV in the only commercially available cell line derived from Atlantic salmon, called the Atlantic salmon (AS) cell line (Flow Laboratories, catalogue no. 02–776, European Cell Collection, Irvine, Scotland, UK). Many viral glycoproteins, e.g. influenza virus haemagglutinin (HA), are activated by proteolytic cleavage (Klenk & Rott, 1988). This is known to make infection possible or enhance the virus yield in cell cultures by mediating multi-cycle replication (Klenk et al., 1975). ISAV inoculation of the AS cells was therefore done with or without trypsin present. The yield of infectious ISAV produced was initially determined by in vivo infectivity trials in groups of salmon. TEM studies gave additional information about the amount of virus, its formation and morphology. A preliminary assumption that ISAV is an orthomyxovirus has previously been made based on biophysical and morphological observations (Sommer & Mennen, 1996). Influenza viruses have the ability to agglutinate erythrocytes from fowl, guinea-pig and human blood group O (Murphy & Webster, 1990). There are few reports of haemagglutinating activity among fish viruses, in contrast to viruses of mammals. Orthomyxovirus-like agents isolated from eel haemagglutinated chicken and sheep erythrocytes, but not those of human, rabbit or rainbow trout (Nagabayashi & Wolf, 1979; Neukirch, 1985). ISAV haemadsorbing ability was studied using infected AS cells and Atlantic salmon erythrocytes. In order to visualize a non-cytopathic ISAV infection in the AS cells, and to quantify the viruses, a haemadsorption centre assay was developed.
A stock of salmon-grown virus was prepared from plasma obtained from experimentally infected ISA-diseased Atlantic salmon. The ISAV was crudely purified from the plasma pool obtained from experimentally infected ISA-diseased Atlantic salmon. The ISAV was harvested after two different periods of time from three identically virus-inoculated AS cell cultures, without trypsin added (AS: 2 days and AS: 11 days) or with trypsin (AS+T: 11 days). Infectivity is shown as cumulative ISA mortality registered in three groups of Atlantic salmon injected with harvested material.

Infectivity is shown as cumulative ISA mortality registered in three groups of Atlantic salmon each weighing about 40 g. The fish were anaesthetized, marked with Alcian blue and given i.p. injections of 0.5 ml each. A group of fish injected with non-infected AS cells and EMEM was always included to monitor mortality due to infection with ISAV released from injected cohabitants. All groups were kept together in one tank and given a continuous flow of brackish water of 10,000 salinity at 10 °C.

The yield of infectious ISAV increased with the incubation time of inoculated cells. This was demonstrated in the in vivo infectivity trials by a significantly increased cumulative mortality and the earlier start of an ISA outbreak in the group of Atlantic salmon injected with material collected 11 days after inoculating the cells, compared to the harvest at day 2 (Fig. 1). There was no significant difference between the amount of infectious ISAV produced with or without trypsin present (Fig. 1). Enhancement of influenza virus infectivity by trypsin has been reported at concentrations of 5 to 20 μg/ml, incorporated in an agar overlay (Tobita et al., 1975; Klenk et al., 1975), or down to 1 μg/ml in the culture medium (Boycott et al., 1994). The AS monolayer detached easily from the wells if exposed to a trypsin concentration exceeding 5 μg/ml for several hours or 1 μg/ml in the medium for several days without FBS present.

Salmon-grown ISAV probably contains fully cleaved spikes and is therefore not dependent on trypsin for a complete single replication cycle in AS cells. Likewise, influenza virus grown in embryonated eggs is highly infectious, while virus produced in some cell cultures has a low infectivity (Klenk et al., 1975; Boycott et al., 1994). Some avian influenza virus strains are cleaved by proteases present in practically all cells. These viruses are therefore capable of undergoing multiple replication cycles in tissue culture, and are highly pathogenic, causing systemic infection in birds (Klenk et al., 1975). The difficulty of finding suitable cell cultures for growing ISAV does not indicate a similar proteolytic cleavability as for the avian stains. ISAV was produced in primary infected AS cells without addition of trypsin, as demonstrated by in vivo infectivity trials. Further studies will show whether AS-produced ISAV requires addition of an exogenous protease for successful in vitro replication.
ISA virus multiplication and haemadsorption

Fig. 2. For legend see facing page.
passages, or if the spikes are already adequately cleaved by an endogenous protease, or will be cleaved at the stage of entry.

AS cells, both non-infected and ISAV-infected, were fixed 11 days after inoculation using 1% glutaraldehyde and 4% formaldehyde in phosphate buffer, 220 mOSm, and embedded in Epon/Araldite. Thin sections of pelleted cells were stained for 1.5 h with 2% aqueous uranyl acetate solution (Nylund et al., 1995), and stained for 12 min with Reynold’s lead citrate before examination in a JEOL JEM 1010 transmission electron microscope. Numerous virus particles somewhat variable in size and form were seen. Generally, they are spherical or ovoid with a mean diameter of 90–130 nm, but filamentous forms of similar diameter occur (Fig. 2a, b). In Fig. 2(c) the ISAV envelope is visible with evenly distributed surface spikes, about 10 nm long, and within the envelope lies an electron-dense matrix shell. The central zone contains amorphous material of variable density, often with a circular arrangement of distinct ‘granulas’ of about 10 nm. This may indicate an organized packing of the nucleocapsid. Structures resembling helical nucleocapsids of 8–10 nm in diameter have previously been detected in purified ISAV material (Sommer & Mennen, 1996).

Monolayers of AS cells grown in six-well cell culture dishes were infected as described above. After incubation at 12 °C for 3 h or 1, 2 or 6 days, a haemadsorption centre assay (HACA) was performed. A suspension of 0.05% (v/v) Atlantic salmon erythrocytes in PBS was added to the cells (1 ml per well) and incubated at room temperature for 45 min. After washing off the erythrocytes not attached to infected cells, HACA-positive cells were observed under a Leitz inverted light microscope. No HACA-positive cells were seen in non-infected cells or in cells 3 h (Fig. 3a) or 1 day after ISAV inoculation, but the first sign of haemadsorption was detected after 2 days of infection. After 6 days the HACA-positive areas were larger and easy to read (Fig. 3b). Usually, to quantify the infectious titre of a virus sample, serial 10-fold dilutions were inoculated onto monolayers of AS cells grown in 96-well cell culture dishes. After incubation for 7 days ISAV infection was demonstrated by use of HACA performed as described above, and TCID₅₀ titres were calculated according to the method of Reed and Muench (Dulbecco, 1980). The titre of the seed virus preparation used for primary infection of the AS cells was 10⁸.₆ TCID₅₀/ml, giving an m.o.i. of about 0.05.

The HACA was used to study possible inhibition of growth by the DNA synthesis inhibitor 5-bromo-2-deoxyuridine (BrdU), a thymidine analogue, and the DNA-dependent RNA synthesis inhibitor actinomycin D (AMD). Virus replication was not inhibited in the presence of 50 µg/ml BrdU, with or without 50 µg/ml thymidine added, indicating that ISAV has an RNA genome. AMD is known to inhibit replication of influenza virus, although this is an RNA virus (Barry et al., 1962). Both nuclear and cytoplasmic transcription
of influenza virus were totally inhibited by 1 µg/ml AMD (Stephenson & Dimmock, 1975). Another proposed orthomyxovirus, isolated from eel, was inhibited most efficiently by 1 µg/ml AMD when treated during the first 2–3 h after infection (Nagabayashi & Wolf, 1979). When AS cells were treated with AMD for 3 h after ISAV adsorption, virus replication was almost completely inhibited by 1 µg/ml (10^3 TCID<sub>50</sub>/ml reduced to < 10 TCID<sub>50</sub>/ml), but not significantly by 0.1 µg/ml AMD. Replication of the double-stranded RNA virus IPNV was not affected by either inhibitor.

Similar to the orthomyxo-like eel virus, the ISAV spikes are somewhat shorter than influenza virus HA. Except for this and the typical ‘granulas’ ISAV is similar in size and morphology to other orthomyxoviruses (Murphy & Webster, 1990). The typical packing of the nucleocapsid is not commonly seen among orthomyxoviruses, but has been reported for paramyxoviruses such as respiratory syncytial virus (RSV) (Norrbys et al., 1970). Nevertheless, it is unlikely that ISAV is an RSV because these viruses show no haemadsorption ability. In addition, the paramyxoviruses are nearly twice as large as ISAV and are not sensitive to AMD. So far, the most obvious differences from other known orthomyxoviruses are that ISAV is pathogenic for a poikilothermic aquatic species and has a much lower optimum growth temperature. A significant reduction in virus production when the growth temperature exceeds 18 °C has been observed (A.-I. Sommer, unpublished results). The present results, including the newly discovered haemadsorbing ability, suggest that ISAV might be an aquatic orthomyxovirus, although placement of ISAV in any specific virus family must await characterization of the viral RNA. The reported propagation of ISAV in a commercial continuous fish cell line will make it easier to complete such studies.

We would like to acknowledge the technical assistance of Randi Olsen* and Helga-Marie Bye* in preparing the material for electron microscopy, and Professor Terje Traavik for critically reading the manuscript (*Institute of Medical Biology, University of Tromsø, Norway). This study was financially supported by the Norwegian Research Council.

References


Received 13 November 1996; Accepted 18 March 1997