Ultrastructure and Sequential Development of Infectious Pancreatic Necrosis Virus

L. HOWARD MOSS III¹ AND MANETH GRAVELL

Laboratory of Virology, St. Jude Children's Research Hospital, and Department of Microbiology, University of Tennessee Medical Units, Memphis, Tennessee 38101

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The morphology and sequential development of infectious pancreatic necrosis (IPN) virus, a pathogen of trouts, were studied by electron microscopy. Mature virions were seen in the cytoplasm of infected cells incubated at 24 C as early as 6 hr after infection. These virions were hexagonal in profile and approximately 55 nm in diameter. Generally between 8 to 10 hr after infection, virus crystals of various sizes were occasionally observed. Although virus replication did not appear to be confined to a particular cytoplasmic locus, mature virions were sometimes seen in association with unidentified tubular structures approximately 45 nm in outside diameter. Negative stains of virus revealed unenveloped icosahedra approximately 65 nm in diameter with probably 92 capsomeres. Contrary to a previous communication which reported IPN virus to have picornavirus-like morphology, we found it to morphologically resemble members of the reovirus group.

Infectious pancreatic necrosis (IPN) virus has been shown to be the etiological agent of an acute, contagious and high-mortality disease of young hatchery-reared trouts (21). Its significance as a trout pathogen in nature has not been determined. Although 8 years have elapsed since its reported isolation and in vitro cultivation (21), only one report has been made regarding its size and morphology (1). Phosphotungstic acid (PTA)-stained IPN virus particles were reported to be unenveloped and cubically symmetrical with an average particle diameter of 18.5 nm. Furthermore, platinum-palladium shadowed particles were found to range between 25 and 29 nm in diameter. Although insufficient resolution of negatively stained particles was achieved to directly study virus fine structure, a photographic image rotation technique purported to reinforce radial periodicity was used to obtain information suggesting that IPN virus is an icosahedron composed of 12 capsomeres. Based upon these morphological characteristics, coupled with its reported cytoplasmic site of synthesis, nucleic acid type (ribonucleic acid), and ether stability, IPN virus was tentatively classified as a member of the picornavirus group (10-12, 19).

This paper presents the results of an electron microscopic study of the morphology and sequential development of IPN virus. These morphological data do not support the conclusion that

IPN virus is a picornavirus, but rather, indicate it to resemble members of the reovirus group.

MATERIALS AND METHODS

Cell culture. Three continuous cell lines originating from poikilothermic vertebrates, fathead minnow (FHM; 7), rainbow trout gonad (RTG; 20), and bluegill (BGL; M. Gravel and R. G. Malsberger, *unpublished data*) cells were used in this study. Stock cultures of these cells were grown at 24 C in 75-cm² plastic flasks (Falcon Plastic, Los Angeles, Calif.) in Eagle's minimum essential medium with Earle's salts (5), supplemented with fetal calf serum to a final concentration of 10% (MEM-10). Maintenance medium was the same except that the serum concentration was reduced to 2% (MEM-2). All media contained 100 units of penicillin, 100 µg of streptomycin, and 50 µg of kanamycin per ml.

Virus. Two isolates of IPN virus were used in this study. Tissue culture passaged stock virus prepared in stationary cultures of RTG cells was used in most experiments (21). Confluent RTG monolayers in 75-cm² plastic flasks were infected with an input virus multiplicity of approximately 0.1 plaque-forming unit (PFU) per cell. The virus inoculum was adsorbed to cells for 1 hr at 24 C, and then 15 ml of MEM-2 was added per culture. Cultures were incubated at 24 C until advanced cytopathology was observed, usually 2 days postinfection. Cell-fluid harvests were subjected to ultrasonic vibration (10-kc Raytheon sonic oscillator) for 1 min to release cell-associated virus (CAV), and then cell debris was removed by centrifugation at 1,000 × *g* for 10 min at 4 C. The supernatant fluid, representing stock virus, was placed in ampoules in small portions and stored at -70 C until used.

¹ Present address: Department of Microbiology, Wayne State University School of Medicine, Detroit, Mich. 48207.

Infectivity titers of stock virus were generally about 10^8 PFU/ml.

The other source of IPN virus was a visceral homogenate of an infected rainbow trout supplied through the courtesy of Kenneth E. Wolf of the Eastern Fish Disease Laboratory, Kearneysville, W. Va. This isolate had never been passed in tissue culture.

Virus purification. Stock virus, prepared as described above, was shaken for 1 min at 0°C with an equal volume of trifluorotrichloroethane (Genesolv-D, Allied Chemicals, Morristown, N.J.). After centrifugation at $600 \times g$ for 10 min at 4°C, the upper aqueous layer containing the majority of virus was separated from the lower fluorocarbon layer. Virus was concentrated by centrifugation at 30,000 rev/min for 1 hr (no. 30 rotor) in a Spinco Model L ultracentrifuge. Pellets were suspended in pH 7.2, 0.14 M phosphate-buffered saline (PBS; 13) containing 0.1% fraction V bovine serum albumin by sonic treatment for 30 sec. Concentrated virus (1 ml) was layered on a 4-ml linear 8 to 40% (w/w) potassium tartrate gradient (pH 7.4) and banded by centrifugation at 36,000 rev/min for 90 min (Spinco Model L centrifuge, SW39 rotor). Bands were collected with a needle and syringe by puncturing the tube wall.

Virus infectivity assay. IPN virus infectivity was determined by plaque assay with FHM cell monolayers. Confluent cultures in 60-mm plastic petri dishes were inoculated with 0.1 ml of virus diluted in PBS containing 2% fetal calf serum. The virus inoculum was adsorbed to cells for 1 hr at 24°C, and then a 1-ml overlay of MEM-2 plus 0.7 to 0.9% agar was added per culture. When the agar overlay had hardened (5 to 10 min), 4 ml of MEM-2 without agar was added per culture. Cultures were incubated for 2 days at 24°C in a 5% carbon dioxide in air atmosphere. The medium-agar overlay was then decanted, cultures were stained with crystal violet, and plaques were enumerated.

Virus growth curve and sequential development. IPN virus growth curves were obtained by infecting monolayers of RTG cells in 60-mm plastic petri dishes (1.35×10^6 cells/dish) with 0.2 ml of tissue culture grown IPN virus at an input multiplicity of 5 PFU/cell. After a 1-hr virus adsorption period, the inoculum was removed, and cultures were washed three times with 5 ml of PBS. Cultures then received 5 ml of MEM-2, and incubation was continued at 24°C in a 5% carbon dioxide and 95% air atmosphere.

At selected intervals after infection, samples were withdrawn for infectivity assay of released virus (RV) and CAV. Samples for determination of released virus were obtained by removing the overlay medium from an infected culture and by centrifuging at $600 \times g$ for 10 min to remove cell debris. Cell monolayers from cultures previously sampled for RV were washed with 5 ml of PBS, 5 ml of MEM-2 was added per culture, and the cell monolayer was dislodged from the culture dish with a rubber policeman. Cells were disrupted by sonic treatment for 1 min, and CAV was assayed.

Confluent monolayers of RTG, FHM, and BGL cells were used for electron microscopic study of the sequential development of IPN virus. Cultures were infected and handled essentially as described above.

Thin sections and negative stains. Thin sections of infected and uninfected cells were prepared for electron microscopy as described previously (3, 4) by fixation in glutaraldehyde, postfixation in osmic acid, and embedding in Maraglas. For negative stains, one drop of virus suspension was placed on a carbon-coated copper grid and allowed to stand for 1 min. The excess fluid was drained off, and the grid was stained with 2% PTA (pH 7.2) for 1 min. The grids were examined immediately in a Siemens Elmiskop I electron microscope using double-condenser illumination at 80-kv accelerating voltage.

Antiserum. Antigens used in the immunization procedure consisted of low-speed clarified fluids from IPN virus-infected RTG cells. Procedures for preparation of IPN virus antigen were as described previously. Preimmunization blood samples were removed from the marginal ear vein of adult rabbits (New Zealand whites), and five 1-ml injections (each containing 10^8 PFU of IPN virus) were administered intravenously over a 5-week period. After the initial injection, a 2-week interval was allowed prior to the second injection. Subsequent injections were given at weekly intervals. Rabbits were bled from the marginal ear vein 1 week after the last antigen injection. Sera were adsorbed with RTG cells to remove antibodies to cellular antigens.

Another IPN antiserum preparation was kindly made available by Richard G. Malsberger, Lehigh University, Bethlehem, Pa. This antiserum was prepared by a procedure similar to that described above.

RESULTS

Virus growth curve. A growth curve showing the kinetics of IPN virus multiplication in RTG cells at 24°C is illustrated in Fig. 1. An increase in CAV was detected between 5 and 6 hr after infection, with an increase in RV occurring 2 to 3 hr later. An exponential increase in infectious virus occurred between 5 and 12 hr after infection. Upon termination of this growth curve 24 hr after infection, yields of CA and RV were approximately equal. These results agree closely with those previously reported by Malsberger and Cerini (11).

Electron microscopy. The temporal and morphological aspects of IPN virus replication were indistinguishable by electron microscopy in the three cell lines used in this study, RTG, FHM, and BGL. Subsequent discussion will apply equally to results obtained in each of the three cell lines.

In agreement with the IPN virus growth curve, virus particles (Fig. 2) were first observed approximately 6 hr after infection. Virus crystals were not seen early in infection, but were occasionally observed later, usually between 8 to 10 hr after infection (Fig. 3 and 4). However, the presence of virus crystals, regardless of time after infection, was not the usual finding. More typical was the finding of individual or small clusters of virus

FIG. 1. Representative growth curve of IPN virus in RTG cells at 24°C. RV indicates released virus (●); CAV indicates cell-associated virus (▲).

particles randomly distributed throughout the cytoplasm (Fig. 2).

Virus particles were found exclusively in the cytoplasm of infected cells, but did not appear to be confined to any particular cytoplasmic locus. In ultrathin sections of infected cells, virions were hexagonal in appearance and had an average diameter of 55 nm.

Generally, virus particles, whether observed intra- or extra-cellularly, were uniformly electron dense, and particles without cores were infrequently observed. Virions were unenveloped and were not seen in association with cell membranes. Furthermore, there was no detectable difference in the diameter of cell-associated and released virions. These findings suggest that virus maturation does not occur at cell membranes.

Primarily between 5 to 7 hr after infection, doughnut-shaped particles, 45 nm in diameter and smaller than mature virions, were seen in the cytoplasm of RTG, FHM, and BGL cells (Fig. 5). Large, isolated bundles of tubules of the same diameter as these particles were also seen (Fig. 6). The doughnut-shaped and tubular structures are believed to be identical and to vary in appearance according to cellular orientation and their plane of sectioning. These structures were sometimes

observed in association with virus particles (Fig. 7). Similar structures were not observed in non-infected cell culture controls.

Negatively stained preparations of virus revealed unenveloped, cubically symmetrical particles with an average particle diameter of approximately 65 nm. Although capsomeres appear to be present on the capsid surface (Fig. 8a, b), we were unable to directly determine their number even though a variety of negative stains were tried. Nonetheless, it appears that four capsomeres are present on a capsid edge, suggesting that the IPN virus capsid is made up of 92 capsomeres. Negatively stained particles with the above morphology were observed both in a visceral homogenate from an IPN-infected rainbow trout and in tissue culture passaged virus. Furthermore, after isopycnic centrifugation of IPN virus in a linear 8 to 40% potassium tartrate gradient, typical 65-nm size virions were detected by negative staining at the same buoyant density as was virus infectivity. Particles with virus-like morphology were not found in other regions of the gradient.

Identification of virus. Because of the discrepancy between our results and those reported previously regarding the size and morphology of IPN virus, neutralization tests were performed to verify the identity of virus isolates used in this study. Antisera prepared independently in this and another laboratory (see Materials and Methods) were tested against two separate isolates of IPN virus; one of which consisted of a visceral homogenate from a rainbow trout with typical symptoms of infectious pancreatic necrosis disease. Greater than 90% neutralization, as determined by plaque reduction, was observed when approximately 10⁷ PFU of either virus isolate was mixed with a 1:200 dilution of either antiserum. Preimmune serum did not significantly neutralize virus infectivity. We conclude from this experiment that virus isolates used in this study were, in fact, IPN virus.

DISCUSSION

IPN virus was previously described as a picornavirus with 12 capsomeres and an average particle diameter by negative staining of 18.5 nm (1). We find IPN virus particles to be unenveloped icosahedra, but by negative staining to be approximately 65 nm in diameter and to most likely have 92 capsomeres. Thus, our results indicate IPN virus to be reovirus-like in size, morphology, and ultrastructure. Although we cannot explain the discrepancy between our results and those of other investigators, we believe our findings are valid for the following reasons. (i) Thin sections of infected RTG, BGL, and FHM cells showed hex-

FIG. 2. FHM cell 9 hr after infection with IPN virus. Note the random distribution of the mature virus particles within the cytoplasm. Nucleus protrudes from the lower left. $\times 45,000$.

FIG. 3. IPN virus crystal in FHM cell 9 hr postinfection. Nucleus is at upper right. $\times 48,000$.

FIG. 4. Unusually arranged IPN virus crystal in FHM cell 9 hr post-infection. $\times 72,000$.

FIG. 5. Small packets of doughnut-shaped particles in RTG cell 6 hr after infection with IPN virus. $\times 50,750$.
 FIG. 6. Bundles of tubular structures of same width as doughnut-shaped particles in Fig. 4. Tubules can be seen in oblique (asterisk) and longitudinal (arrow) cross section. BGL cell 7 hr after infection with IPN virus. $\times 50,000$.
 FIG. 7. BGL cell 8 hr after infection with IPN virus. Note the close proximity of tubular structures to mature IPN virus particles. $\times 50,000$.

agonal particles, sometimes grouped in crystalline arrays, which are approximately 55 nm in diameter. The morphology and time of appearance of these particles in infected cells of these three lines were indistinguishable. Particles with similar morphology were not detected in uninfected RTG, BGL, and FHM cells. (ii) By negative staining, unenveloped, cubically symmetrical particles approximately 65 nm in diameter were seen both in harvests of tissue culture passaged virus and in a visceral homogenate of a rainbow trout with typical symptoms of IPN disease. (iii)

FIG. 8. Negative stains (PTA) of extracellular IPN virus particles. (a) Full IPN virus particle. Four capsomeres can be seen on each of two edges of the capsid. $\times 300,000$. (b) Empty IPN virus particle. Four capsomeres can clearly be counted on one edge. A suggestion of capsomeres can be seen in the central portion of the particle. $\times 300,000$.

IPN virus antisera prepared in two different laboratories were effective in neutralizing the infectivity of both IPN virus isolates used in this study. (iv) Unenveloped icosahedral particles approximately 65 nm in diameter were found at the same buoyant density in linear potassium tartrate gradients as was infectivity. Particles with virus-like morphology were not detected in other regions of the gradient. (v) IPN virus growth curves shown herein are in agreement with those reported previously by other investigators (11). Although we did not detect a picorna-like virus in fluids or cells from IPN virus-infected cultures, the presence of such a virus cannot be excluded with absolute certainty. However, cytopathology observed in our infected cell cultures agrees with that described previously (10), and appears to originate from the reo-like virus we described.

IPN virus replication appears to occur exclusively within the cytoplasm of susceptible cells. A specific site of virus replication was not found, but mature virions were sometimes seen in association with tubular structures. Although we have not attempted to identify these tubular structures, similarities exist between them and those described by Dales (2) in reovirus type 3-infected L cells. Reovirus type 3 was shown to replicate in perinuclear inclusions in frequent association with spindle tubules. Spindle tubules of uninfected L cells were 18 to 23 nm in outside diameter, but in reovirus-infected cells, in many instances, these tubules were coated with a dense material about 20 nm in thickness. Immunofluorescent staining of this dense material suggested it to be viral capsid protein (15, 16). Although tubular structures seen in IPN virus-infected cells appear too large (45 nm in outside diameter) to be spindle tubules, coating of spindle tubules with viral antigen could account for their size. An alternative explanation is that tubular structures found in IPN virus-infected cells are aberrant virus forms originating through errors in assembly (6, 8, 9, 18, 22).

Reoviruses have been recovered ubiquitously from man and many lower vertebrates (17). In spite of their widespread distribution, their significance as human or animal pathogens is little understood. Reovirions are ether-stable, unenveloped icosahedra that range in size from 60 to 75 nm. Among the plant-insect viruses, wound tumor and rice dwarf viruses have similar characteristics. Reo-like viruses are unique in that a high proportion of their genome is composed of ribonucleic acid in the double-helical configuration (14). We find that IPN virus resembles reo-like viruses in size, morphology, and ultrastructure. Preliminary data also indicate that IPN

virions contain double-stranded ribonucleic acid (M. Gravel, *unpublished data*). Although these results are strongly suggestive, additional studies are required to definitely place IPN virus within the reovirus category.

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