Characterization of EV-2, a Virus Isolated from European Eels (*Anguilla anguilla*) with Stomatopapilloma

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A virus designated EV-2 has been isolated from external tumor tissue and internal organs of European eels (*Anguilla anguilla*) with stomatopapilloma. It contains RNA and is ether, acid, and temperature labile above 4°C, and concentrated preparations agglutinate chicken and sheep erythrocytes. The addition of actinomycin D during the first 2.75 h of infection inhibits viral replication. As determined in sucrose gradients, the buoyant density of the virus is 1.19 g/cm³. EV-2 has a moderately pleomorphic spherical morphology; its diameter ranges from 80 to 140 nm. The virion has narrow, regularly spaced surface projections about 10 nm long. Replication in FHM cells at 15°C shows new infectivity appearing at 10 h postinfection and reaching a plateau at 20 h. Cytopathic effects consist of cell fusion, syncytia, and irregularly rounded cell masses. Viral antigen was detected in the cytoplasm of infected cells by specific immunofluorescence.

Some European eels (*Anguilla anguilla*) are afflicted with epidermal growths occurring predominantly about the mouth and head and consisting of hyperplastic squamous cells in papillomatous array (1, 9). The condition was first reported in 1947 by Christiansen and Jensen (2), who described the tumor as having a cauliflower-like appearance. Following the lead of Schäperclaus (14), later investigations referred to the neoplasms as "cauliflower disease of eels." In 1969, Pfitzner and Schubert (11) reported the isolation of an icosahedral virus from blood of affected eels. Four companion papers were published on the eel neoplasm: Pfitzner (10) described in vitro studies of her isolate, Schubert (16) reported on electron microscopy, Schmid (15) gave histopathological findings, and Koops and Mann (8) discussed the epizootiology. Although details were not given, Deys (3) reported virus in tumorous eels. In Japan, Sano (13) reported an icosahedral virus from European and from Japanese eels (*Anguilla japonica*).

Our study was undertaken to characterize a virus isolated by Wolf and Quimby (18) from European eels with stomatopapilloma.

MATERIALS AND METHODS

Viruses and cells. EV-2 was plaque purified from a culture that had been isolated from a tumor-bearing European eel by Wolf and Quimby (18) and designated EV-1. Other viruses used were American Type Culture

Collection (ATCC) strains of infectious pancreatic necrosis virus (VR-299), channel catfish virus (VR-665), and infectious hematopoietic necrosis virus (VR-714).

We used the following certified cell lines of the ATCC: CCL-42 (FHM), CCL-55 (RTG-2), CCL-59 (BB), and CCL-91 (BF-2). Stocks of EV-2 were prepared only in FHM cells.

Cells were grown in Eagle minimal essential medium (Earle balanced salt solution) with 10% fetal bovine serum (MEM-10). Maintenance medium consisted of the same minimum essential medium without serum (MEM) or with 5% fetal bovine serum (MEM-5). The medium used for plaque assay contained 0.016 M Tris buffer (pH 7.6) and 2% fetal bovine serum (MEM-2 Tris). All media contained 100 U of penicillin, 100 µg of streptomycin, and 25 U of nystatin per ml. We quantified virus by end-point dilution in tube or microwell cultures or by plaque assay, using a procedure described elsewhere (19). Plates were incubated at 15°C for 6 days before fixation and staining.

We used standard procedures to determine sensitivity to lipid solvents, and determined the effect of freeze-thaw cycles with a dry ice-acetone bath. Stability to sonic disruption was tested with a Heat Systems Ultrasonics, Inc., cell disruptor, model W185D, and a microtip with an indicated 55 W of energy. Suspensions of human (O+), sheep, rabbit, chicken, and rainbow trout (*Salmo gairdneri*) erythrocytes were standardized to 0.5% in phosphate-buffered saline at pH 7.2 and tested at room temperature or at 4°C for agglutination by crude concentrations of EV-2. Viruses of influenza and Newcastle disease were used as positive controls.

EV-2 was concentrated and purified in the following manner. Debris was removed from infective cell culture medium by low-speed centrifugation and filtration through a 0.45-µm membrane. Virus was then

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pelleted by centrifugation at $55,000 \times g$ for 1 h at 4°C in a Spinco no. 30 rotor. The resulting virus pellets were resuspended in MEM, MEM-2 Tris, or Hanks balanced salt solution and then applied to a linear 15 to 60% (wt/wt) sucrose gradient and centrifuged at $52,000 \times g$ for 3 h at 4°C in a Spinco SW25.1 rotor.

Antiserum was prepared in rabbits, which received an initial intramuscular injection of a homogenate of equal parts of concentrated virus and Freund complete adjuvant and a later subcutaneous booster injection several days before terminal bleeding. The serum was adsorbed with an equal volume of FHM cells and clarified by centrifugation.

Presumptive identification of viral nucleic acid type was made by determining the effect of several concentrations of 5-iodo-2-deoxyuridine or 5-bromo-2-deoxyuridine (BUdR) on the replication of EV-2 by FHM cells. Control cultures were grown in medium without

inhibitor or in medium with inhibitor plus thymidine. Effects of actinomycin D were determined by treating cells at room temperature before infection, during adsorption, or after adsorption, or (in some tests) by continuous exposure at 15°C .

Electron microscopy. Virus preparations were applied to carbon-coated collodion or Formvar grids, negatively stained with neutralized phosphotungstic acid, and then examined with a JEM 100 B electron microscope. The technique of Kapikian et al. (6) was used to prepare virus samples for direct immune electron microscopy.

Light microscopy. We observed FHM cells infected with EV-2 by an indirect fluorescent antibody technique, using fluorescein isothiocyanate-conjugated goat anti-rabbit serum (Cappell Laboratories Inc., Cochranville, Pa.). FHM cells infected with EV-2 were also fixed in Carnoy solution and stained with

FIG. 1. One-step growth curve of EV-2 at 15°C in FHM cells infected at a multiplicity of infection of 10 PFU per cell. (—) Absence and (+) presence of cytopathic effects.

0.01% acridine orange in citrate-phosphate buffer (pH 3.8). Some samples were treated with 0.05% RNase for 30 min at 25°C or with 0.02% pepsin for 10 min at 25°C after the RNase digestion.

RESULTS

Virus growth and cell susceptibility. Of the four fish cell lines tested, EV-2 replicated only in FHM cells. The range of temperature for growth was 10 to 25°C; optimal replication occurred at 15°C. At 20°C, the yield of virus was only one-half that at 15°C and at 10°C only about one-third. Adsorption of EV-2 to FHM cells was rapid; 5 min of adsorption at 20°C resulted in maximal plaque numbers.

A one-step growth curve employing a multiplicity of infection of 10 showed a lag phase of about 8 h during which infectivity declined and cytopathic effects were not evident (Fig. 1). New infectivity appeared at about 10 h, and exponential growth followed for about 5 h. Cytopathic effects first became apparent during the exponential growth phase and were maximal at 72 h after infection. Released virus and cell-associated virus both attained levels of about $10^{5.3}$ PFU/ml at 20 and 30 h, respectively. Infectivity of released and cell-associated virus declined in parallel until about 72 h, when they apparently stabilized.

Infection of FHM cells at 15°C resulted in syncytia formation and irregularly rounded cell masses, and infected cells eventually lysed. At 20 and 25°C, cytopathic effects were restricted to pyknosis and lysis.

Biological properties. EV-2 was completely inactivated by exposure to chloroform, ether, or acid pH (pH 3.0), but was stable at pH 6.0 to 9.0. Three cycles of freezing and thawing did not reduce infectivity. Sonic disruption at 55 W for up to 120 s did not reduce infectivity.

EV-2 was decidedly temperature labile (Table 1); at 4°C, 99.7% of the infectivity was lost by day 15. At 15°C, comparable loss occurred within 4 days, and at 25°C no residual infectivity could be detected at 30 and 48 h. Heating at 50°C for 10 min resulted in complete inactivation, and the effect was not prevented by the presence of 1 M $MgCl_2$. Inasmuch as EV-2 was somewhat unstable at -20°C, lower temperatures should be used for long-term storage. Similarly, lyophilization resulted in a great loss of infectivity, and we do not know how long the residual infectivity persists.

Differential filtration through serum-treated membranes showed that infectivity passed filters of 220-nm mean pore diameter but was retained by 100-nm membranes.

TABLE 1. *Virus infectivity titer at three temperatures*

Storage period (days)	Titer ($\log_{10}$ PFU/ml) at temp (°C):		
	4	15	25
0	6.3	6.1	6.1
0.25	ND ^a	ND	4.9
0.5	ND	ND	3.6
1	ND	5.4	2.4
1.25	ND	ND	1.0
2	6.0	4.7	ND
3	ND	3.8	ND
4	5.9	3.3	ND
5	ND	2.9	ND
6	5.7	2.7	ND
7	ND	0	ND
8	5.1	ND	ND
10	5.0	ND	ND
12	4.6	ND	ND
15	3.8	ND	ND

The peak of infectivity of EV-2 in a sucrose gradient corresponded to a density of 1.19 g/cm³ (Fig. 2).

Fluorescence microscopy. The indirect fluorescent antibody technique revealed the accumulation of virus-specific products in the cytoplasm of fused and rounded cells. Brightly fluorescent cytoplasmic granules were seen early in the infection, but nuclei were never fluorescent. Staining with acridine orange revealed yellow to yellow-green cytoplasmic granules only in infected cells, and these granules were unaffected by RNase or by pepsin followed by RNase. Enzyme treatments obliterated the usual bright orange of control cell cytoplasm.

Electron microscopy. Particles about 90 to 140 nm in diameter with an outer structure consisting of rod-shaped projections about 10 nm long were observed regularly in infective culture medium (Fig. 3). Additional studies were made by immune electron microscopy to determine whether these particles reacted with rabbit anti-EV-2 serum. Reaction of virus with antiviral serum resulted in the appearance of aggregates of viral particles that appeared to be coated with antibody (Fig. 4). The particles were not randomly distributed but were present as groups that stood out clearly from surrounding matter. The particles from these aggregates were about 60 to 80 nm in diameter and showed a membrane-like structure (Fig. 4A). Another aggregate, probably consisting of disrupted virus particles, showed very fragile membrane-like structures and some inner components (Fig. 4B). Roughly spherical structures (about 35-nm diameter) were also observed (Fig. 4C). No aggre-

FIG. 2. Isopycnic centrifugation of EV-2 in sucrose density gradient. A 1-ml sample of virus was layered onto a 15 to 60% (wt/wt) linear sucrose gradient prepared in TNE buffer (0.01 M Tris buffer, pH 7.6, 0.1 M NaCl, and 0.001 M EDTA). The gradient was centrifuged at $52,000 \times g$ for 14 h at 4°C in a Spinco SW25.1 rotor. Fractions (about 1 ml each) were collected by piercing the bottom of the centrifuge tube. The density of each fraction was calculated from the refractive index, and viral infectivity was quantified by plaque assay.

FIG. 3. Three particles of EV-2, negatively stained with phosphotungstic acid. Particles were spherical or slightly pleomorphic and were surrounded by rod-shaped surface projections. No internal structure was evident.

FIG. 4. *Negatively stained aggregates of EV-2 particles. The virus suspension was incubated with rabbit anti-EV-2 serum prior to preparation for electron microscopy. (A) Most particles were 60 to 80 nm in diameter and were spherical with a membrane-like structure. Some inner components were visible, but surface projections (see text) were not. The three particles at left resembled untreated virus; one was pleomorphic and, at 190 nm in greatest dimension, was exceptionally large. (B) Degenerated particles, about 50 nm in diameter, were abundant and showed no surface projections. (C) After 4 days at 4°C, roughly spherical structures (about 35 nm in diameter) of unknown significance were also observed.*

gates were observed in control preparations of the virus.

Effects of metabolic inhibitors. Neither 5-bromo-2-deoxyuridine ($10^{-3.5}$ or $10^{-3.8}$ M) nor 5-iodo-2-deoxyuridine (10^{-4} M) inhibited the replication of EV-2 (Table 2).

Regardless of when it was applied to FHM cells, actinomycin D ($5 \mu\text{g/ml}$) resulted in almost complete inhibition of EV-2 synthesis; the results were most pronounced during the 2.75-h period immediately after infection (Table 3).

TABLE 2. Infectivity titers of EV-2 replicated in the presence of three metabolic inhibitors

Inhibitor and concn	Titers on virus controls ^a		
	EV-2	IPNV (RNA)	CCV (DNA)
5-Bromo-2-deoxyuridine			
0	5.1	≤ 7.5	4.5
$10^{-3.8}$ M	5.1	≤ 7.5	0
$10^{-3.8}$ M + $10^{-3.4}$ M thymidine	5.1	ND	4.5
$10^{-3.5}$ M	5.1	≤ 7.5	0
$10^{-3.5}$ M + $10^{-3.3}$ M thymidine	5.1	ND	4.5
5-Iodo-2-deoxyuridine			
0	4.5	≤ 6.5	5.5
$10^{-4.0}$ M	4.8	≤ 6.5	≤ 0.5
$10^{-4.0}$ M + $10^{3.3}$ M thymidine	4.5	≤ 6.5	5.5
Actinomycin D			
0	4.1	ND	7.0
$5 \mu\text{g/ml}$	0	ND	3.0

^a Culture medium was harvested on day 4 of incubation. Titers expressed as $\log_{10}$ 50% tissue culture infective dose. IPNV, Infectious pancreatic necrosis virus; CCV, channel catfish virus. ND, Not done.

TABLE 3. Infectivity titers of EV-2 replicated in FHM cells^a

Time when treated (h)	Titer at actinomycin D concn ($\mu\text{g/ml}$) ^b		
	5.0	1.0	0.5
Before inoculation			
0.75	1.1	4.6	4.5
During adsorption	1.7	4.4	4.5
After inoculation			
0.75	0	1.2	3.5
1.75	0	2.2	4.0
2.75	0	2.6	4.1
6.75	1.7	4.1	4.3

^a FHM cells were treated with actinomycin D at various times and assayed on day 3 of incubation. Infectivity of the untreated culture was 10^5 PFU/ml.

^b Titer expressed as $\log_{10}$ PFU per milliliter.

When actinomycin D ($1 \mu\text{g/ml}$) was used before or during adsorption, it effected a 60 to 80% inhibition of EV-2 replication. However, like the $5\text{-}\mu\text{g}$ level, the effects of $1 \mu\text{g/ml}$ were greatest during the 2.75 h after infection, and at the very least there was a 99.6% inhibition. Although the effects were less, the pattern of inhibition also occurred at $0.5 \mu\text{g/ml}$.

Hemagglutination. When concentrated to 10^6 PFU/ml or greater, EV-2 agglutinated chicken and sheep erythrocytes. End points of virus dilutions were 1:16 to 1:64 with sheep cells and 1:16 to 1:32 with chicken cells. Spontaneous elution occurred in tests that were done at room temperature and in plates that were moved from 4 to 20°C .

DISCUSSION

From its biophysical properties, the effects of 5-bromo-2-deoxyuridine and 5-iodo-2-deoxyuridine on its replication, and the reactions of infected cells to acridine orange, we conclude that EV-2 has an RNA genome. From its size and shape, ability to agglutinate at least some erythrocytes, and sensitivity to actinomycin D, we consider EV-2 to have the general characteristics of an orthomyxovirus (5, 12, 17). Specific placement of EV-2 in any virus group must await characterization of the viral RNA.

As originally described (18), the source culture of the virus used in our study produced cytopathic effects both in FHM and in RTG-2 cells, but EV-2 was not replicated by RTG-2 cells. In contrast, the virus reported by Pfitzner and Schubert (11) showed lytic cytopathic effects in RTG-2 cells at 16 to 18°C , and the agent itself was clearly icosahedral and had a diameter of 52 to 56 nm. Sano (13) also reported an icosahedral virus from *A. anguilla* and *A. japonica* in Japan. Sano's agent, EVE, is clearly separable from EV-2 on the basis of morphological and biophysical properties.

We do not know the relationship of EV-2 to the neoplasm of the original host eels. In young eels (20 to 30 cm long) obtained from Delaware's coastal waters for use in experimental infections with EV-2, virological examination of a subsample showed that about 20% harbored an agent with properties similar to those of EV-2. However, the virus from the North American eels was not neutralized by rabbit antiserum against EV-2. In contrast, elvers about 7 cm long from waters off America's northeast coast were apparently free of detectable virus. When injected with about 3×10^5 PFU of EV-2, about half of the elvers died within 3 months, but virus could be recovered from only about 25% of the dead eels. Further study is necessary to determine the

relationship of EV-2 to EV-1, to the eel itself, and to the uncharacterized North American and Japanese isolants.

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