Identification and Characterization of a Second Feline Herpesvirus

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Received for publication 21 June 1973

A feline virus isolate previously reported as adenovirus-like has more recently been identified and characterized as a new feline herpesvirus. This herpesvirus, which appears to be cell associated, has some characteristics of the cytomegaloviruses and has been found to induce chemical crystal formations in infected cell cultures.

In previous investigations of viral induced feline uro lithiasis, a virus was isolated which appeared to be unlike any of the known feline viruses. It produced a variety of cytopathic effects (CPE) in cell cultures and intranuclear inclusions suggestive of an adenovirus. Additionally, CPE, noted in cell cultures, included the formation of syncytial cells, enlarged cells, apparent genetic changes, and the formation of intra- and extracellular chemical crystals (5). This virus now has been identified as a new feline herpesvirus (NFHV) by electron microscopy, and by serological, physical, and chemical means.

MATERIALS AND METHODS

Preparation of viral stocks. The isolation of the virus and media used were described previously (5). Viral stocks were prepared by inoculation of the stable Crandell feline kidney cell line (CRFK) while the cells were suspended in small volumes of growth medium. The inoculum consisted of 0.3 ml of infected cell culture fluids with sufficient cells to seed a 250-ml tissue culture flask. The virus was allowed to adsorb to the cells for 15 min at 4 C as they were slowly agitated with the aid of a magnetic stirrer. The cells with the adsorbed virus were then transferred to the culture flasks, and the volume of growth medium was adjusted to 40 to 50 ml. The flasks were incubated in a stationary position at 35 C. Since the CPE was not always apparent in uninfected cell cultures, Leighton tube cultures were prepared from each lot of inoculated cells to monitor the optimal time for harvesting the viral stocks. Leighton tube cover slips were stained with a modified May-Grünwald-Giemsa stain. When at least 50% of the cells were observed to have intranuclear inclusions (in 6 to 11 days), the virus stocks were harvested. The infected cell culture fluids were centrifuged in a refrigerated centrifuge at 500 rpm for 10 min to remove cells and cellular debris.

Viral stocks were titered by inoculating 0.1 ml of 10-fold stock dilutions, in maintenance medium, into each of four Leighton tubes at the time of seeding with CRFK cells. The cover slips from these cultures were stained as described above after 6 to 7 days of incubation at 35 C. The titer was calculated by the Spearman and Karber (13) statistical method based on the presence of one or more intranuclear inclusions and expressed as the mean tissue culture infective dose (TCID50).

Two methods were used to increase viral titers. The first involved scraping all residual cells which were then still attached in the flasks into the infected supernatant cell culture fluids. The mixtures were then sonically disrupted with the microprobe of a sonic oscillator as described by Calnek et al. (2) for 30 s. To obtain viral stocks of higher titers, the infected supernatant culture fluids were harvested separately from the residual cells. This supernatant was pelleted at 154,041 x g for 90 min in a Beckman model L-2 ultracentrifuge using an SW39 rotor. The pellets were added to the residual cells and resuspended in 0.1 or 0.01 of the original volume of medium and sonically disrupted as described above.

Cell susceptibility. The infectivity and CPE of the virus were observed in a variety of cells such as primary feline kidney (PK1), embryonic feline lung (FEL), dog kidney cells (DK), HeLa cells, and embryonic bovine heart (BEH), lung (BEL), and spleen (BESP) cells. Each of four Leighton tube cell cultures were inoculated with 0.1 ml of virus stock at the time the cells were seeded. This was done for three subpassages. The third passage in the cells was then titered both in the homologous cells and in CRFK cells.

All cell cultures, reagents, and media were monitored for microbial contamination including mycoplasma (1, 7). Only cell cultures and reagents free of contamination were used in these experiments.

Electron microscope techniques. Electron microscopy studies included examination of ultrathin sections prepared from pellets of infected cell cultures as described elsewhere (10, 12, 15). In addition, one lot of concentrated and purified virus was prepared for negative staining with phosphotungstic acid (PTA) and examined with an RCA electron microscope. This virus stock was first concentrated by centrifugation.
The sonicate then was purified by zonal centrifugation in sucrose gradients with densities between 1.35 to 1.20 g/cm². Gradients were centrifuged in the same manner used for the concentration of the viral stock. The bands were collected, washed with sterile distilled water, and repelleted twice to remove the sucrose. The pellets were resuspended in sterile distilled water corresponding to 1:100 of the original volume of infected cell culture fluids. In preparation of viral stock for the PTA staining, it was found that the NFHV stock purified by zonal centrifugation banded at a density of 1.28 g/cm² in the sucrose gradients.

Ether and chloroform sensitivity. The ether sensitivity for one viral stock was tested by the method of Hamparian et al. (9). A portion of untreated sample of the same viral stock was handled identically. After the treatment, both samples were titered for viral concentration.

The chloroform sensitivity was tested by the Feldman and Wang method (8). Both chloroform-sensitive and -resistant viruses were used as controls in this test. The viruses used as controls were: the C27 feline herpesvirus (3) and the FJ and Manx feline caliciviruses (formerly called feline picornaviruses). The feline syncytium-forming viral isolate 383 (FSFV 383) also was included.

Nucleic acid determination. The nucleic acid determination was made by the Dart and Turner (4) modification of the acidine orange stain. Both ribonucleic acid (RNA) viruses (FJ and Manx feline caliciviruses) and a deoxyribonucleic acid (DNA) (C27 feline herpesvirus) virus were included as controls. Also tested at the same time was the FSFV 383 isolate.

Serological techniques. Serum neutralization (SN) test methods were described previously (11). The only modification was the use of Leighton tube cultures to facilitate examination for inclusion bodies. A variety of feline viruses, including the C27 feline herpesvirus, feline panleukopenia, and FSFV, as well as a number of other human and animal herpesviruses, including infectious bovine rhinotracheitis, pseudorabies, herpes simplex, canine herpes, bovine mammalitis, and monkey B, were compared with NFHV.

Fluorescent-labeled antibodies (FA) for the C27 feline herpesvirus, infectious bovine rhinotracheitis, and pseudorabies virus, as well as the feline panleukopenia virus and feline syncytium-forming virus, were used to stain infected cell cultures from Leighton culture tubes. (The fluorescent-labeled antibodies were kindly supplied by Donald Gustafson, Purdue Univ., Lafayette, Ind.) The methods used for conjugating the antibodies with fluorescein, fixation, and staining of the cover slips have been described in the text by Thompson and Hunt (14).

Microhemagglutination tests (MHA) as modified by D. W. Holmes (personal communication) substituted a 1% normal rabbit serum for phosphate-buffered saline (PBS) to avoid autoagglutination of the feline red blood cells (RBC) in PBS. Equal volumes of virus dilutions and 5% RBC (0.025 ml each) were mixed and incubated at room temperature in sealed microtiter plates for 90 min. The C27 feline herpesvirus was used as a positive control.

Production of antiserum to NFHV. Two conventionally reared adult female cats were test bled and found to be negative for SN antibodies to NFHV, but unfortunately antibodies to C27 feline herpesvirus were found, so their usefulness was limited. The cats were given 1.0 ml of NFHV intravenously (i.v.) with a viral titer of TCID₅₀ 4.4 per 0.1 ml. At 21 days they were test bled and given a second inoculation of 0.3 ml of the same virus stock intramuscularly (i.m.). They were inoculated in a similar manner with the same amount of virus at weekly intervals thereafter for a total of eight i.m. inoculations.

The two cats were test bled at intervals during this period. After 9 months from the initial i.v. inoculation, the cats were inoculated with an additional 0.3 ml of virus stock i.m. One week later they were anesthetized, bled, killed, and autopsied.

RESULTS

Viral titers. To date primary isolations of the NFHV has only been made from autogenous cell cultures initiated from infected cat tissues. The difficulty experienced in production of cell-free virus stocks with measurable titers hampered the early studies. An eclipse phase, in which no intranuclear inclusions or other CPE were observed in inoculated cell cultures, was the primary difficulty encountered. Infectious virus could be demonstrated in these cultures when cultures were trypsinized and reincubated. These observations indicated that the virus was cell associated. Viral titers of the supernatant stocks had end points of TCID₅₀ between 10¹ and 10², and rarely 10³/0.1 ml. The titers of viral stocks prepared by sonically disrupting the supernatant and residual cells varied from a TCID₅₀ of 10² to 10⁴/0.1 ml. The concentrated supernatant and residual cell sonicate stocks had viral end points between a TCID₅₀ of 10⁴ to 10⁴/0.1 ml.

CPE. A variety of CPE have been observed in NFHV-infected cell cultures in autogenous heart, bladder, and kidney cell cultures and also in the CRFK cell cultures. The cytopathic changes included the condensation and reticulation of the nuclear chromatin, often with bizarre nucleolar changes. The number of nucleoli varied from one to four, and on occasion more were seen. An unusual change was noted in many nucleoli. These often assumed the configuration of Chinese letters. In addition, an increase in the numbers of mitotic figures was seen. Preliminary chromosomal studies (kindly performed by H. E. Dunn of the N.Y. State Veterinary College, Cornell Univ.) in CRFK-infected cells indicated that the number as well
as the size, arrangement, and structure of the chromosomes was abnormal.

A variety of intranuclear inclusions was noted in cell cultures. Some were similar in appearance to adenovirus inclusion bodies, others looked like atypical panleukopenia inclusions, whereas still others were intensely basophilic with a marked granular appearance. None of the inclusion bodies resembled those produced by the C27 feline herpesvirus.

Syncytial cells, enlarged cells, and transformed cells that lost contact inhibition have been observed in many of the infected monolayer cell cultures. Infected cell sheets maintained at 35°C have remained attached to culture flask surfaces for periods ranging from 4 to 8 months without addition of fresh media. In contrast, cell sheets of uninoculated cell cultures detach from the surface in 4 to 8 weeks.

In addition to the variety of CPE noted in cell cultures infected with NFHV, this virus has been observed to induce several types of chemical crystals both intracellularly and extracellularly (5). Recently, one of these crystal types has been identified as cholesterol (6).

**Susceptibility of various cell types.** It was found that infection could be established in three types of feline cells (FK1°, FEL, and CRFK) and in the bovine embryonic cells (BEH, BEL, and BESP). Intranuclear inclusions as well as various other types of CPE were seen in these infected cell cultures. After the second passage of NFHV in these cells it was found that the FK1°, FEL, and BEH control cell cultures were not maintaining and, since fresh cells were unavailable, the use of these cells was discontinued. However, the virus was passed a third time in the remaining cell types. Titers in homologous as well as CRFK cells of this passage are given in Table 1.

It appeared that the primary DK and HeLa cells were resistant to the NFHV infection. Only rare intranuclear inclusions were seen in the first passage, whereas in the second passage only one intranuclear inclusion was seen in one of four cover slips of inoculated and stained DK Leighton tube cell cultures. No inclusions were seen in the third subpassage in either of these cell types. In addition, other types of CPE seen in feline of bovine cells were not noted in the DK or HeLa cells.

**Electron microscopy.** The examination of ultrathin sections of a NFHV-infected CRFK cell culture revealed the typical intranuclear array and morphology of a herpesvirus as illustrated in Fig. 1. Figure 2 illustrates the cubic symmetry of the naked herpesvirus in the negative PTA stain. The average diameter of 10 particles measured on the negative of this micrograph was approximately 115.5 nm.

**Ether and chloroform sensitivity.** The titer of one viral stock of NFHV after ether treatment was found to be TCID₅₀ of 10^8.9 ± 0.1 ml as compared to a viral titer of TCID₅₀ of 10^8.9 ± 0.1 ml for the untreated control. The results of the chloroform sensitivity of NFHV as compared to C27 feline herpesvirus, FJ and Manx caliciviruses, and FSFV 383 are summarized in Table 2. The results indicate that the NFHV, C27, and FSFV strains are sensitive to chloroform, whereas the two feline calcivirus strains, FJ and Manx, are resistant.

**Identification of the nucleic acid.** The nucleic acid of NFHV was identified as DNA with the acridine orange stain as was the C27 feline herpesvirus. In the same test the FSFV, Manx, and FJ virus strains were shown to be RNA viruses. These results are summarized in Table 3.

**SN tests.** Twofold dilutions of antiserum to various animal herpesviruses including C27 feline herpesvirus, infectious bovine rhinotracheitis virus, canine herpesvirus, bovine mastitis virus, pseudorabies virus, monkey B virus, and herpes simplex virus failed to neutralize approximately 10^7 TCID₅₀ of NFHV. The lowest dilution used for each antiserum tested was 1:2. The NFHV was neutralized by its homologous feline antiserum. Similarly, it was found that antiserum to infectious canine hepatitis virus (canine adenovirus 1), feline panleukopenia virus, feline reovirus, and FSFV 383 did not neutralize the new herpesvirus. These results are summarized in Table 4.

In the MHA tests with two stocks of NFHV, one of which was concentrated, no hemagglutination was observed in any dilution. The C27 feline herpesvirus used as the positive control had an MHA titer of 1:4.

**Response in cats to NFHV.** One of the two female adult cats developed a measurable SN titer after a single i.v. injection of NFHV followed by three i.m. injections at weekly

### Table 1. Comparative titers of the new feline herpesvirus in bovine embryonic cells and in the CRFK cell line

<table>
<thead>
<tr>
<th>Cells</th>
<th>Viral titer* (TCID₅₀/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homologous cells</td>
</tr>
<tr>
<td>CRFK</td>
<td>4.8</td>
</tr>
<tr>
<td>BEK</td>
<td>5.5</td>
</tr>
<tr>
<td>BESP</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* After third viral passage in respective cell cultures.
Fig. 1. Electron micrograph of an ultrathin section of an infected CRFK cell culture revealing the typical herpesvirus morphology and array of the new feline herpesvirus viral particles within a cell nucleus. ×65,880.

intervals. The titer of the antiserum was 35 on the 35th day after the first viral injection, and it did not rise significantly thereafter.

At autopsy, no lesions were found in the cat which did not show an antibody response. However, the cat which did have an SN titer rise was found to have an enlarged and extremely hemorrhagic ovary. NFHV was reisolated from the autogenous ovary cell cultures but not from the heart, bladder or kidney autogenous cell cultures of this cat.

DISCUSSION

The results of our studies to characterize and identify the new viral isolate clearly place it in the herpesvirus group rather than the adenovi-
Fig. 2. Electron micrograph of the concentrated (100x) and purified new feline herpesvirus revealing the cubic symmetry of the unenveloped herpesvirus. The average diameter of several particles was approximately 115.5 nm. Phosphotungstic acid negative stain. ×65,880.
rus group as suggested earlier (5). Based upon neutralization and fluorescent-antibody tests, the virus has been found to be serologically distinct from any mammalian herpesviruses, including the C27 feline herpesvirus, to which it has been compared. Its pathological effects in cell culture resemble changes characteristic of cytomegaloviruses, clearly different from the CPE observed for the C27 feline herpesvirus. In addition, the new herpesvirus is more cell associated and produces lower viral titers in cell culture than the C27 feline herpesvirus. The changes produced in infected cell cultures suggest that this virus is similar to the cytomegaloviruses.

Preliminary evidence suggests that the NFHV may induce antibody production in cats, and additional studies are in progress to study the pathological effects of this new herpesvirus.

TABLE 2. Chloroform sensitivity tests of various feline viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Viral titer (TCID₅₀/0.1 ml)</th>
<th>Untreated</th>
<th>Chloroform treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>New feline herpesvirus</td>
<td>4.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>New feline herpesvirus*</td>
<td>3.5</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>C27 feline herpesvirus</td>
<td>5.3</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>FJ strain, feline calicivirus</td>
<td>5.0</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Manx strain, feline calicivirus</td>
<td>5.3</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>383 strain, feline syncytium-forming virus</td>
<td>3.5</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

* Represent two different viral stocks.

TABLE 3. Nucleic acid identification of the new feline herpesvirus and of strain 383 feline syncytium-forming virus*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Color reaction with AO alone</th>
<th>Color reaction after treatment with:</th>
<th>Nucleic acid type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer alone</td>
<td>DNase</td>
<td>RNase</td>
</tr>
<tr>
<td>New feline herpesvirus</td>
<td>Yellow-green*</td>
<td>Yellow-green</td>
<td>Yellow-green</td>
</tr>
<tr>
<td>C27 feline herpesvirus</td>
<td>Yellow-green*</td>
<td>Yellow-green</td>
<td>Yellow-green</td>
</tr>
<tr>
<td>Strain 383, feline syncytium-forming virus</td>
<td>Flame red*</td>
<td>Flame red</td>
<td>Flame red</td>
</tr>
<tr>
<td>FJ strain, feline calicivirus</td>
<td>Flame red*</td>
<td>Flame red</td>
<td>Flame red</td>
</tr>
<tr>
<td>Manx strain, feline calicivirus</td>
<td>Flame red*</td>
<td>Flame red</td>
<td>Flame red</td>
</tr>
</tbody>
</table>

* The neutralizing antibody titer is expressed as the reciprocal of the 50% end point as calculated by the Spearman and Karber method (13). Twofold serum dilutions beginning with a 1:2 dilution of serum were used for all the serum neutralizations.

* In general, the virus titers in the respective tests were approximately TCID₅₀ x 10⁴.

* Kindly supplied by National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.

* By the acridine orange (AO) stain with the feline herpesvirus (C27) as the DNA control and two feline caliciviruses (FJ and Manx) as the RNA controls.

* Yellow-green fluorescence most intense in the nucleus.

* Absence of yellow-green fluorescence, DNA digested by deoxyribonuclease (DNase).

* Flame-red fluorescence most intense in the cytoplasm.

* Absence of flame red fluorescence, RNA digested by ribonuclease (RNase).
These observations introduce a new dimension in viral studies. The potential role of such viral effects in the pathogenesis of urolithiasis and degenerative vascular diseases should be considered.

ACKNOWLEDGMENTS

These studies were supported by the Morris Animal Foundation and by the Public Health Service General Research Support Grant PR 05462-09 and PR 05462-10 from the Division of Research Facilities and Resources.

We would like to acknowledge the assistance of various members of the professional and technical staff of the New York State Veterinary College at Cornell University. We especially thank J. Fabricant for monitoring all our cell cultures and reagents for mycoplasmal contamination as well as for his valuable criticism in preparing the manuscript. We acknowledge the expertise of R. W. Dellers, J. H. Carlson, and R. D. Schultz for their assistance with the fluorescent antibody studies. R. W. Dellers also performed the comparative serum neutralizations for the bovine mammalitis virus. We also are grateful to D. F. Holmes for performing the microhemagglutination tests and to F. W. Scott for helping to differentiate the NFHV in early studies from the feline panleukopenia virus.

We are indebted to Loretta Bates and Louise Barr for their assistance in the electron microscopy studies. Carol Hoff and Nancy Aagaard also rendered invaluable technical assistance.

LITERATURE CITED