Studies on Cytopathogenic Viruses from Cats with Respiratory Infections

III. Isolation and Certain Properties of Feline Herpesviruses

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Abstract. Six strains of feline herpesvirus were isolated alone or in association with the feline picornavirus from eight domestic cats showing the respiratory syndrome. All of them agglutinated feline erythrocytes. Their hemagglutinating activities were enhanced by treatment with ethyl ether. These strains could not be distinguished from one another by the serum-neutralization test.

Biological and physicochemical properties of feline herpesvirus were investigated. The status of feline viral respiratory infections in Japan was also discussed.

Up to date, feline picornavirus (FPV) and feline herpesvirus (FHV) have been implicated mainly in the clinical entity of feline respiratory infections.

Since the first isolation of FPV in 1957 [12], many investigations have been made in various parts of the world. Moreover, it has been understood that there are many serotypes of FPV, as examined by the serum-neutralization (SN) test.

On the other hand, in 1958 FHV was first isolated from kittens showing the upper respiratory syndrome by Crandell and Maurer [7], and the name "feline viral rhinotracheitis" (FVR) was proposed for the disease by Crandell and Despeaux [5]. This original isolate, designated "C-27", is the prototype strain of FHV [28]. Since then, FHV was isolated in Switzerland [3], Canada [9], Hungary [16], Great Britain [18], France [13], New Zealand [2] and Australia [26]. In company with these isolations, many clinical, virological, immunological and pathological studies on FVR have been reported.

In Japan, some case reports have been made with emphasis put on pathological findings [10, 17, 25]. The present report deals with further details of the virological characteristics of the authors' own FHV strains.

Materials and Methods

Cell cultures and media

Secondary feline kidney cells (FKC-2) and lined feline lung cells (FLC) (Fig. 3) were used for virus isolation and/or routine virus assay. FKC-2 culture was examined for contamination with any other virus before use.

The growth medium used for both cultures consisted of Eagle's minimum essential medium (Eagle's MEM) supplemented with 10% tryptose phosphate broth (TPB) and 10% inactivated calf serum (CS). Maintenance medium consisted of Eagle's MEM supplemented with 10% TPB and 2% CS. Antibiotics were added to both media to give a final concentration of 200 units of penicillin...
G potassium, 200 μg of streptomycin sulfate, 100 μg of kanamycin and 50 units of nystatin per ml.

Virus strains

Virus isolation trials were conducted on eight domestic cats showing the respiratory syndrome. The virus isolation technique and identification procedures used for feline picornaviruses were the same as previously described [19, 27]. The C16 strain, supplied by Dr. C. Papageorgiou, of the Institut Mérieux, Lyons, France, was used as a reference strain of FHV [13].

Virus titration

Serial tenfold dilutions were prepared from the stock virus fluid with Tris-buffered magnesium saline [29]. Then 0.2 ml of aliquot was inoculated into four tubes per dilution. Usually, 5 to 7 days after inoculation, the end point was determined by the appearance of a typical CPE (Fig. 3). The titer was expressed as the 50% tissue culture infective dose per 0.2 ml (TCID50/0.2 ml) by the method of Reed and Muench [24].

Physicochemical characteristics of the virus

The C7301 strain was used for experiments. Its nucleic acid type was determined and its sensitivity to ether, low pH (3.0) and heating (50°C, 30 minutes) examined by the procedures described previously [27]. The filtration test was performed with Millipore filters 450, 220, 100 and 50 nm in pore size.

Electron microscopy

FLC inoculated with the C7301 strain were detached from the glass surface with a rubber policeman 24 hours after inoculation, and centrifuged at a low speed for five minutes. The resulting pellets were fixed in a mixture of 4% paraformaldehyde and 5% glutaraldehyde with 0.2 M sodium cacodylate buffer. Then they were postfixed in 1% osmium tetroxide, dehydrated through the ascending concentrations of ethanol, transferred to propylene oxide, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead nitrate, and examined by the Hitachi HU-12 electron microscope.

In addition, negative staining with phosphotungstic acid (PTA) was carried out. Infected FLC cultures were frozen and thawed. The resulting cell suspension was clarified by centrifugation at 5,000 rpm for 30 minutes. The supernatant thus obtained was centrifugated at 20,000 rpm for 40 minutes at 4°C. The pellet was resuspended in such amount of sterile distilled water as corresponding to 1:100 of the original volume of infected cell culture fluid. A small amount of the resulting virus suspension was mixed with an equal volume of 2% PTA adjusted to pH 7.0. The mixture was in touch with a collodion-coated grid. Excess fluid blotted dry, the grid was examined by the electron microscope.

Hemagglutination (HA) activity

The HA test was performed by the tube method with equal volumes of a twofold dilution of the virus and 0.5% feline erythrocyte suspension in phosphate-buffered saline solution (PBS) at pH 7.5. The HA titer was expressed as the reciprocal of the highest virus dilution showing complete HA. In the test to determine the condition of HA, the following items were investigated: (a) temperature (4, 20 and 37°C), (b) pH (6.5, 7.5 and 8.5), (c) correlation of infective titer to HA titer, and (d) HA activity of the virus after treatment with ethyl ether.

Animal experiment

Two healthy, sero-negative kittens approximately two months old were inoculated with 0.2 ml (10^4.9 TCID50/0.2 ml) of the FHV C7301 strain intranasally. During the observation period after inoculation, rectal temperature and clinical signs were recorded daily, and attempts made to recover the virus from the nasal, ocular and oropharyngeal regions.

Immune sera

Anti-C16 strain immune serum was prepared from two rabbits by the method described by Studert and Martin [26]. A rabbit was inoculated intravenously with 5 ml (10^4.0 TCID50/0.2 ml) of the C7301 strain weekly for eight weeks. Serum was collected from it three weeks after the last inoculation.

All the sera were stored at −20°C and inactivated by heating at 56°C for 30 minutes before examination.

Serum-neutralization (SN) test

The SN test was performed by the serum dilution method. Serial twofold serum dilutions were mixed with an equal volume of virus suspension containing 100 to 320 TCID50/0.2 ml and allowed to stand at 4°C for 18 hours. Then 0.2 ml of the mixture was inoculated into each of four FLC culture tubes. After incubation at 37°C for 7 days, the tubes were observed for the appearance of CPE. The SN titer was expressed as the reciprocal of the serum dilution at which CPE had been suppressed 50% of the tubes by using the method of Reed and Muench [24].

Results

Virus isolation

As shown in Table 1, 17 virus strains were isolated from eight domestic cats.
manifesting the respiratory syndrome. Of them, eight were FHV strains and the others FPV strains. FHV and FPV were isolated from the same region of different cats or simultaneously from different regions of one cat.

Physicochemical characteristics of the C7301 strain are shown in Table 2.

Virus replication and hemagglutination

Six isolates of FHV grew well in FLC cultures accompanied with the formation of Cowdry type A intranuclear inclusion bodies and multinucleated giant cells (Figs. 1 and 3).

All the seven strains of FHV grown in both FKC-2 and lined FLC cultures agglutinated feline erythrocytes. Complete HA was observed in the crude virus fluid which had had an infective titer of $10^{6.8} \text{TCID}_{50}/0.2 \text{ ml}$ on an average. Furthermore, as illustrated in Fig. 1, the HA titer was enhanced by treatment with peroxide-free ethyl ether at a concentration of 20% (v/v) at 4°C for 18 hours.

There were no differences in HA titer among pH 6.5, 7.5 and 8.5 of PBSS. The highest reproducible HA titer was obtained at 37°C and with an 0.5% erythrocyte suspension. Therefore, the HA test was performed with PBSS at pH 7.5 and an 0.5% erythrocyte suspension, and reading was made after incubation at 37°C for three hours. HA activity was stable at 37°C, but was lost by heating at 56°C for 30 minutes. There was no difference between crude and ether-treated hemagglutinin in the above-mentioned conditions.

Electron microscopy

In thin sections of infected FLC, viral particles were identified in the nucleus, cytoplasm and extracellular space (Fig. 4). The intranuclear particles ranged from 80 to 96 nm and was 89 nm on an average in
diameter. They appeared round or oval in shape and contained an electron-dense or ring-like nucleoid, which was bound by an outer membrane. The intracytoplasmic particles ranged from 128 to 165 nm and was 140 nm on an average in diameter. They were morphologically identical, but somewhat larger than the intracytoplasmic particles.

In negative staining study, both enveloped and naked particles were observed (Fig. 5). The enveloped particles varied in shape with an average diameter of 178 nm. The capsids of naked particles exhibited a round or hexagonal outline with an aver-
age diameter of 108 nm. Furthermore, particles which were considered to be "tailed" particles were seen.

Clinical signs of experimentally inoculated cats

Rectal temperatures and clinical signs of two cats inoculated experimentally are illustrated in Fig. 2. These cats developed a severe upper respiratory disease characterized by epiphora, sneezing and fever on the 6th to 8th day after inoculation. Nasal and conjunctival discharges were serous during the incipient stage of disease, and became viscid later. Cat No. 0476 was in a very severe condition. In it, dyspnea and anorexia were mostly pronounced before death, which occurred on the 16th day after inoculation. Cat No. 0576 presented milder symptoms than this cat. It exhibited no anorexia. Its clinical conditions began to be improved on the 17th day after inoculation.

Serological relationships among FHV strains

Serological relationships among FHV strains are shown in Table 3. It was not possible to distinguish the seven FHV strains from one another by the SN test.

### Discussion

Six isolates possessed the general physicochemical properties, including electron microscopic features, of the herpesvirus group. When experimentally inoculated with them, cats developed a severe upper respiratory disease. As the clinical signs of spontaneous feline upper respiratory diseases were complicated, it seemed difficult to presume their causal agents. Actually, unexpected viruses were sometimes isolated, as shown in Table 1. This is an interesting finding in itself.

Feline picornavirus infections have been considered to be main viral upper respiratory infections in Japan until recently [19, 27], but FHV has not been implicated in the main causal agent. The results of virus isolation in the present and other studies [10, 17], however, indicate that there have been many cases of FVR in Japan.

Three members of the herpesvirus group have been known to date to possess HA activity. They are equine abortion virus (EAV) [20, 21], avian laryngotracheitis virus [28] and FVR virus [14]. EAV agglutinated horse erythrocytes. In fresh virus harvests HA titers were directly proportional to infective titers, 1 HA unit being equal to about $10^7$ PFU/ml in PK-15 cells. Hemagglutinin appeared to be an integral part of the viral envelope. It was a protein and its activity was not dependent on lipids [20]. On the other hand, there is only one paper published on HA of FHV. This was written by Gillespie et al. [14], who described that the FHV C27, F15 and FH2CS strains had caused HA of feline erythrocytes at a low titer (1:2 to 1:8) only when propagated.
in the primary or secondary FKC culture or in the diploid feline tongue cell line. In the present study, complete HA was observed by the crude virus fluid which had had an infective titer of $10^{7.8}$ TCID$_{50}$/0.2 ml. The highest HA titer was obtained at 37°C. It was considered that FHV might generally possess an HA activity on feline erythrocytes.

FHV can not be propagated to such extent as exceeding an infective titer of about $10^{6.0}$ TCID$_{50}$/ml. Therefore, when frozen and thawed, the crude cell culture fluid is low in HA titer (1:1 to 1:2). This antigen is not used in the HI test with sufficiency. So, it is necessary to prepare a large quantity of virus antigen and to treat it by the purified-concentration technique. This problem could be settled by treating the crude cell culture fluid with ethyl ether in the present study. As for EAV, such lipid solvents as ether and chloroform inactivated infectivity, but did not enhance HA activity [20]. As shown in Fig. 1, hemagglutinin of FHV for the HI test was obtained sufficiently after treatment with ethyl ether.

In a similar way, viral hemagglutinin has been obtained by treatment with a lipid solvent in the study of myxoviruses [15, 22]. For instance, Henry et al. [15] mentioned that the hemagglutinin titers of parainfluenza 4 (A and B) were so enhanced by a Tween-ether treatment that the viruses could be used for the HI test. Hemagglutinin of FHV was not investigated, except for the influence of heating on HA activity, in the present study. It is very interesting to note that FHV showed the same phenomenon as the paramyxoviruses. Further work will be required to determine the nature of hemagglutinin of FHV and to apply it to the serologic test.

Up to date, the SN test has been only one tool for the serological investigation of FHV and its infection [1, 6, 8]. According to Crandell [4], FHV is one immunologic type identified by the SN test, though clinical manifestations are different from one isolate of this virus to another. No detailed studies have ever been carried out on the virulence and antigenicity of such isolates. Six strains of FHV used in the present study were considered to be the same as the prototype strain designated “C-27” of FVR virus [7, 28], but not the same as that of FHV 2 reported recently [11], on the basis of the results of experimental infection in cats, the aspects of CPE in feline cell cultures, and a weak HA activity on feline erythrocytes.

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References


Explanation of Figures

Fig. 3.
A: Normal feline lung cell culture on day 4 after seeding. May-Grünwald-Giemsa (MGG) stain, \( \times 300 \).
B: Early typical CPE presented by the FHV C7301 strain 24 hours after inoculation. MGG stain, \( \times 300 \).
C: Multinucleated giant cells 48 hours after inoculation. MGG stain, \( \times 1200 \).
D: Intracellular inclusion bodies 48 hours after inoculation. MGG stain, \( \times 1200 \).

Fig. 4. Virus particles in thin sections of feline lung cell 24 hours after inoculation with FHV C7301 strain.
A: Overview of infected feline lung cells. Bar=1 \( \mu \)m.
B: Intracellular particles. Bar=100 nm.
D: Extracellular particles. Bar=100 nm.

Fig. 5. Virus particles by negative staining with phosphotungstic acid.
A: Naked particles. Bar=100 nm.
B: Particles considered to be “tailed” particles. Bar=100 nm.
C: Enveloped particles. Bar=100 nm.
FELINE RESPIRATORY VIRUSES. III