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Characteristics of a Virus Isolated from a Feline Fibrosarcoma

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A virus was isolated from a radioresistant feline fibrosarcoma. It induced multinucleated giant-cell formation and lysis in a cell line derived from a canine fibrosarcoma, which was used to characterize the virus. End-point titrations in these cells required 28 days. The virus was sensitive to ether and heat and was destroyed at *p*H 3. Replication was not inhibited by 5-bromodeoxyuridine. Electron microscopy revealed assembly by a budding process from the plasma membrane of infected cells. The average diameter of the virion was 106 nm. Intracisternal particles with an average diameter of 45 nm were present within infected cells. In two instances secondary monolayers of feline renal cells underwent morphological transformation after inoculation of the virus. The two strains of transformed cells are now in continuous culture and do not yield infectious virus.

The majority of the viruses that have been isolated from the cat are picornaviruses (9). The herpesvirus group is represented by feline rhinotrachetitis virus (10, 12, 14, 19, 40). Feline ataxia virus and the major feline pathogen, feline panleukopenia virus, are currently considered to be parvoviruses (3, 29–32, 36, 53). A feline reovirus has recently been described (F. S. Scott, 1970, Amer. J. Vet. Res., *in press*). Taxonomic characteristics of the isolates of oncogenic feline viruses are limited at present to density, size, and morphology (26–28, 34, 35, 37, 38, 43, 44, 46, 48). The virus characterized in this study differs from all viruses previously isolated from cats (3, 4, 7, 11, 13, 15, 20, 21, 45, 54).

The virus was isolated from a fibrosarcoma which occurred in a 5-year-old, domestic shorthair, castrated male cat. The animal underwent two attempts at surgical excision of the tumor as a house pet in Jacksonville, Fla. It was subsequently referred to The Ohio State University Veterinary Hospital where rapid growth of the tumor continued after two implantations of iridium 192. Approximately 4 months after the neoplasm was detected the cat was killed. At necropsy the tumor was a lobulated mass which infiltrated the left infraspinatus and supraspinatus muscles and measured 20 by 15 by 15 cm. Its cut surface had a mucinous character.

Tissue samples of the tumor were collected aseptically at necropsy for in vitro cultivation, viral isolation, and histopathology. An attempt to cultivate the tumor cells resulted in progressive degeneration and cellular death during 3 subpassages over an interval of 52 days. Virus was subsequently isolated from all pools of culture medium collected at 9 different intervals during the attempt to cultivate the tumor cells. The virus was also isolated from a 10% suspension of the original tumor tissue prepared in Hanks balanced salt solution (HBSS).

MATERIALS AND METHODS

Virus. The pool of virus used in these studies was prepared from the third subpassage of the virus in a cell line derived from a canine fibrosarcoma. Cell-culture medium and infected cells were harvested 12 days postinfection. The pool was frozen at −90 C and subsequently thawed, redistributed in portions, refrozen, and maintained in liquid nitrogen vapor. This pool of virus was titrated 27 times over an interval of 27 months. The average titer was $10^{5.5}$ 50% tissue culture infectious doses ($TCID_{50}$)/ml. The infectivity titer did not decrease during storage.

Canine herpesvirus (CHV) was used as a control deoxyribonucleic (DNA) virus in studies of the effect of 5-bromodeoxyuridine on virus replication. CHV was obtained from Leland E. Carmichael, Veterinary Virus Research Institute, Cornell University, Ithaca, N.Y. The DNA character of CHV was established previously (1, 6, 41, 47).

Cell culture. A cell line derived from a canine fibrosarcoma was used as the indicator system in isolation and characterization studies of the virus.

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The cell line had been in continuous culture in this laboratory for more than 4 years. It was transferred at 2-week intervals through 104 subcultures. Subculture 27 was used to isolate the virus, and subsequent subcultures were used for characterization studies. The titer of the characterized virus pool was checked 27 times between subcultures 36 and 98 and no alteration in susceptibility occurred.

Electron microscopy and animal inoculation studies indicated that the canine cell line was free of viral infection. Virus-like particles were not observed in ultrathin sections of biopsy specimens of the original tumor. Cell cultures of the tumor tissue including subcultures 27 and 95, among others, did not contain virus particles that were detectable by electron microscopy (Fig. 1). Viable cells from the original culture and various subcultures from 1 through 72 were inoculated by different parenteral routes into neonatal Syrian hamsters, weanling BALB/c mice, neonatal conventional Chihuahua dogs, neonatal gnotobiotic Beagle dogs, and into cheek pouches of cortisone-treated weanling Syrian hamsters. No lesions, neoplastic or otherwise, which were referable to the inoculated cells resulted.

Repeated examinations for mycoplasma contamination of the cell line and the pool of virus isolate were negative. Positive control cultures of a feline mycoplasma isolate obtained from David L. Madden, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, were used in these tests. The laboratory of Leonard Hayflick, Stanford University, Stanford, Calif., confirmed the absence of mycoplasma contamination of subculture 100 of the canine cell line.

Titration of virus. Tenfold dilutions of the virus were made in HBSS. After removal of medium, each of five 7-day-old, confluent, Leighton-tube cultures were inoculated with 0.2 ml of virus and incubated for 1 hr at 37 C. Fresh medium was then added and changed twice weekly thereafter. Five cultures were sham-inoculated with HBSS to serve as control cultures for each series of titrations. End-point titrations required a minimum of 28 days of observation. Viral infection was detected by a cytopathic effect (CPE) characterized by an initial multinucleated giant-cell formation (Fig. 2) followed by lysis. Virus titer was calculated by the method of Reed and Muench (42) and expressed as $TCID_{50}$.

Growth curve. Growth of virus in the canine fibrosarcoma cell line was followed daily for 21 days. Leighton-tube cell cultures were each inoculated with $10^{3.8}$ $TCID_{50}$. Each day thereafter, medium was collected from two tubes and frozen. Both cells and medium from two other tubes were also collected by exposure to three freeze-and-thaw cycles (dry ice and acetone). All harvests of virus were stored at −60 C for subsequent titration of virus content.

Exposure to ether. The technique of Andrewes and Horstman (2) was modified as previously described (39) to expose the virus to a concentration of 20% ether by gentle agitation for 20 hr. A portion of the same suspension of virus served as a control and was treated in the same manner by using double-distilled deionized water in lieu of ether. The virus was titrated immediately after treatment.

Exposure to chloroform. Virus was exposed to 4.8% chloroform for 20 min in the same manner as it was exposed to ether.

Exposure to heat. Two 3-ml samples of virus were sealed in 5-ml serum vials. One was placed in a water bath heated to 50 C, and the other was maintained at room temperature. Both samples were manually agitated at 5-min intervals for 30-min. Both were titrated for virus content immediately thereafter.

A virus sample which was maintained in a sealed serum vial at 37 C was titrated for virus content on days 1, 3, and 7. Another virus sample maintained at 4 C in similar fashion for 379 days was titrated for virus content on days 4, 114, 226, and 379.

Exposure to pH 3. The *p*H of two equal portions of the virus was adjusted by dropwise addition of 1.0 N hydrochloric acid while the virus suspension was manually agitated. The *p*H was reduced to 3.05 in one portion and to 7.05 in the other. Both samples of virus were maintained at 4 C for 30 min and then titrated for viable virus.

Exposure to 5-bromodeoxyuridine (BUDR). Leighton-tube cultures of indicator cells were incubated in excess of 4 hr with maintenance medium containing 60 μg of BUDR/ml. Control cultures included those incubated with 60 μg of BUDR plus 60 μg of thymidine per ml, and those incubated with maintenance medium only. After incubation, 10 cultures in each of the three groups were inoculated with $10^{3.2}$ $TCID_{50}$ of the viral isolate per culture. A control DNA virus, canine herpesvirus, was inoculated into 10 additional tubes from each of the three groups at a concentration of $10^{4.9}$ $TCID_{50}$/tube. The concentrations of BUDR and thymidine were maintained at each of two medium changes, which were performed prior to termination of the experiment. At 6 and 10 days after exposure, five cultures in each group were harvested and pooled after exposure to one freeze-thaw cycle. The pools were maintained at −60 C for subsequent titration of virus content.

Hemagglutination and hemadsorption procedures. Erythrocytes from chickens, guinea pigs, and type O human blood were used in attempts to demonstrate hemagglutination by the virus isolate. Blood specimens from chickens included those from sexually mature birds as well as three pooled samples from 1-day-old resistance-inducing factor-free chicks. All blood samples were washed three times in physiological saline, and the erythrocytes were used as 1% suspensions. Virus containing $10^{5.5}$ $TCID_{50}$/ml was used undiluted and at twofold dilutions in saline through 1:128. Reaction mixtures were comprised of equal parts of virus suspension, saline diluent, and 1% suspension of erythrocytes. Separate portions of the mixtures were incubated at 4 C, room temperature (24 to 26 C), and 37 C. Results were recorded at 30-min intervals for 2 hr and at 18 hr.

Guinea pig erythrocytes were washed as described and used as 0.5% suspensions in attempts to demonstrate hemadsorption. Inoculated cell cultures in 100-ml prescription bottles which had developed a detectable CPE were rinsed once with HBSS, and 2 ml of the 0.5% suspension of erythrocytes was added to each culture. After incubation at 4 C, cultures were examined for hemadsorption at 30-min intervals for

3 hr. Cultures were tested at 8, 9, 14, 15, 16, 19, and 20 days after virus inoculation.

Virus response to antibiotics. The virus isolate was titrated by using maintenance medium containing 200 μg/ml of kanamycin sulfate (Kantrex) or 7-chlortetracycline hydrochloride (Aureomycin). The medium was added to the 0.2-ml inoculum of virus in each tube immediately after 1 hr of incubation at 37 C.

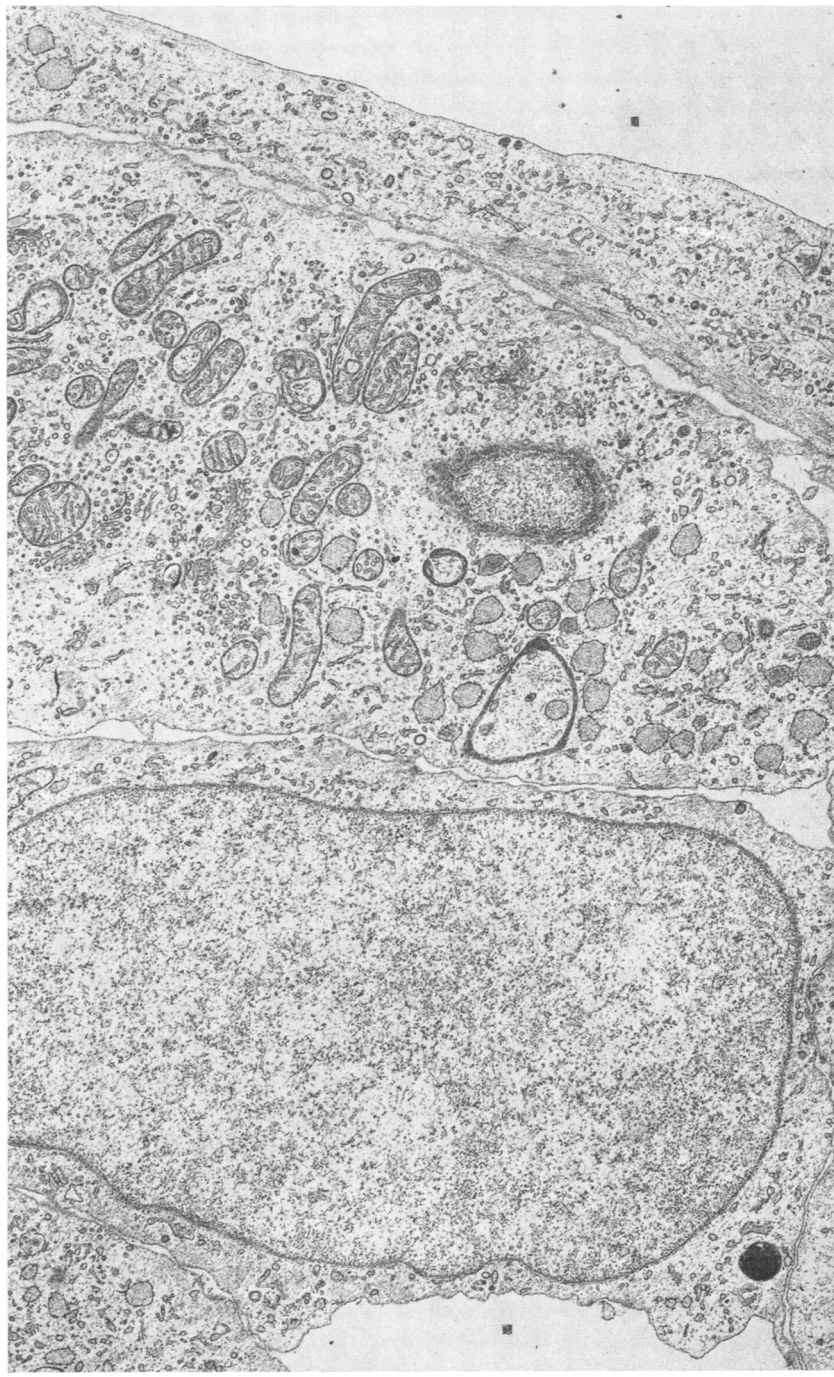

FIG. 1. *Subculture 27 of the indicator cell line derived from a canine fibrosarcoma. Plasmalemma and three different regions of three different cells from a 14-day-old uninfected control culture are apparent. Bar, 100 nm.*

The same medium was freshly prepared and used for each medium change throughout the experiment.

Filtration of virus. Virus suspension was centrifuged at 1,000 × *g* for 15 min at 4 C for clarification. The supernatant fluid was divided into 3.5-ml quantities which were passed through membrane filters once (Millipore Corp., Bedford, Mass.). The membranes were held in Swinnex-25 filter holders (Millipore), and filtration was accomplished by manually applied positive pressure in 10-ml syringes. The average pore diameters of the various filters were 300, 220, 100, and 50 nm. Filitrates were titrated immediately for virus content.

Electron microscopy. Confluent cultures of indicator cells in 100-ml prescription bottles were inoculated with $10^{5.0}$ $TCID_{50}$ of the viral isolate per bottle. At 14 days after inoculation, the cells were fixed in situ for 10 min with osmic acid buffered to *p*H 7.2. The fixed cells were dehydrated by seven sequential exposures to concentrations of ethyl alcohol from 35 to 100%. The dehydrated fixed cells in each bottle were scraped into 5 ml of 100% ethyl alcohol and pelleted by light centrifugation. The pellets were resuspended and infiltrated sequentially by two 5-min exposures to propylene oxide, one 60-min exposure to a 50% solution of propylene oxide in Maraglas, and incubation in Maraglas alone overnight at 4 C. The infiltrated pellets were embedded in fresh Maraglas and incubated for 3 days at 60 C to complete polymerization. Sections were cut at 25 to 30 nm thickness, stained with uranyl acetate and lead hydroxide, and examined with a Philips 200 electron microscope. Uninfected cultures of indicator cells were processed and examined in the same manner.

Virus was prepared for negative staining by partial purification. Two parts of a virus pool were mixed with one part trichlorotrifluoroethane (Genetron 113) which was precooled at 4 C. The mixture was emulsified with a "45" homogenizer (The VirTis Co, Inc., Gardiner, N. Y.) for 5 min at a rheostat setting of 65. The emulsified mixture was separated by centrifugation at 1,550 × *g* for 5 min at 5 C. The aqueous phase of the mixture which contained $10^{5.0}$ $TCID_{50}$/ml of virus was decanted and centrifuged at 85,000 × *g* for 120 min at 5 C. Each pellet was resuspended in phosphate-buffered saline at *p*H 7.4 to a final volume of 0.7 ml, which represented an approximate concentration factor of 165. A drop of the partially purified virus concentrate was placed on a copper grid (300 by 300 mesh) coated with 1% collodion, stained with 1 drop of 1% phosphotungstic acid at *p*H 7.2, drained to filter paper by capillary action for 2 min, and immediately examined with the electron microscope.

RESULTS

In vitro growth of virus. Growth of virus in the canine fibrosarcoma cell line for 21 days is plotted in Fig. 3. Viral replication was evident within 2 days after inoculation. The CPE, however, was not obvious until 7 days after inoculation. Peak concentrations of virus developed between 10 and 14 postinoculation days. At the same time, differences of virus concentration became appreciably less in collected medium as compared to medium and cells combined. Multinucleated giant cells which characterized the CPE became most apparent during this time also (Fig. 2).

The virus also replicated in 10 other cell types representing those of feline, canine, avian, equine, porcine, simian, and human species. Epithelioid and fibrocytic cellular patterns were represented among the various cell types. On the basis of viral replication, susceptibility, and CPE, the canine fibrosarcoma cell line represented the indicator of choice.

Effect of lipolytics on virus. Viable virus was not detectable after exposure to ether or chloroform (Table 1).

Heat lability of the virus. Exposure to 50 C for 30 min destroyed 99.9% of the virus. After 1 week of exposure to 37 C, a suspension of the virus contained $10^{3.1}$ $TCID_{50}$. The infectivity of another suspension was gradually destroyed during storage at 4 C for 376 days (Table 1).

Acid lability of the virus. No viable virus was detectable after exposure to an environmental *p*H of 3.05 for 30 min at 4 C. (Table 1).

Nucleic acid inhibition. Replication of the virus isolate was not inhibited by the incorporation of 60 μg of BUDR in the culture medium for 10 days (Table 2). A similar exposure to BUDR inhibited replication of CHV, a DNA virus. Thymidine reversed the CHV inhibition induced by BUDR. By inference, the nucleic acid of the virus isolate is ribonucleic acid (RNA).

Attempts to demonstrate virus hemagglutination and hemadsorption. Neither hemagglutination nor hemadsorption could be demonstrated for the virus by using the techniques described.

Virus response to antibiotics. Neither kanamycin sulfate nor 7-chlortetracycline hydrochloride at a concentration of 200 μg/ml in the culture medium significantly inhibited replication of virus. Titration values for the virus were $10^{5.3}$ $TCID_{50}$/ml with 7-chlortetracycline hydrochloride, $10^{5.5}$ with kanamycin sulfate, and $10^{5.5}$ without either of the two antibiotic additives.

Filtration of virus. Virus passed through filters with an average pore diameter of 220 nm or larger (Table 1). It did not pass through those with an average pore diameter of 100 nm or smaller.

Electron microscopy of the virus. The virus was assembled at the plasmalemma of the infected cells by a budding process. An electron-lucent nucleoid acquired an envelope as it protruded into a crescent-shaped thickening of the cellular plasma membrane (Fig. 4A). The intact virion was spherical and its average approximate external diameter was 106 nm. The nucleoid of the

virion had an electron-lucent center with an electron-dense border. The approximate average diameter of the nucleoid was 53 nm. In some particles a thin electron-dense membrane was discernible between the nucleoid and the external surface of the virion (Fig. 4A). The average approximate diameter of the portion of the virion enclosed within this membrane was 79 nm.

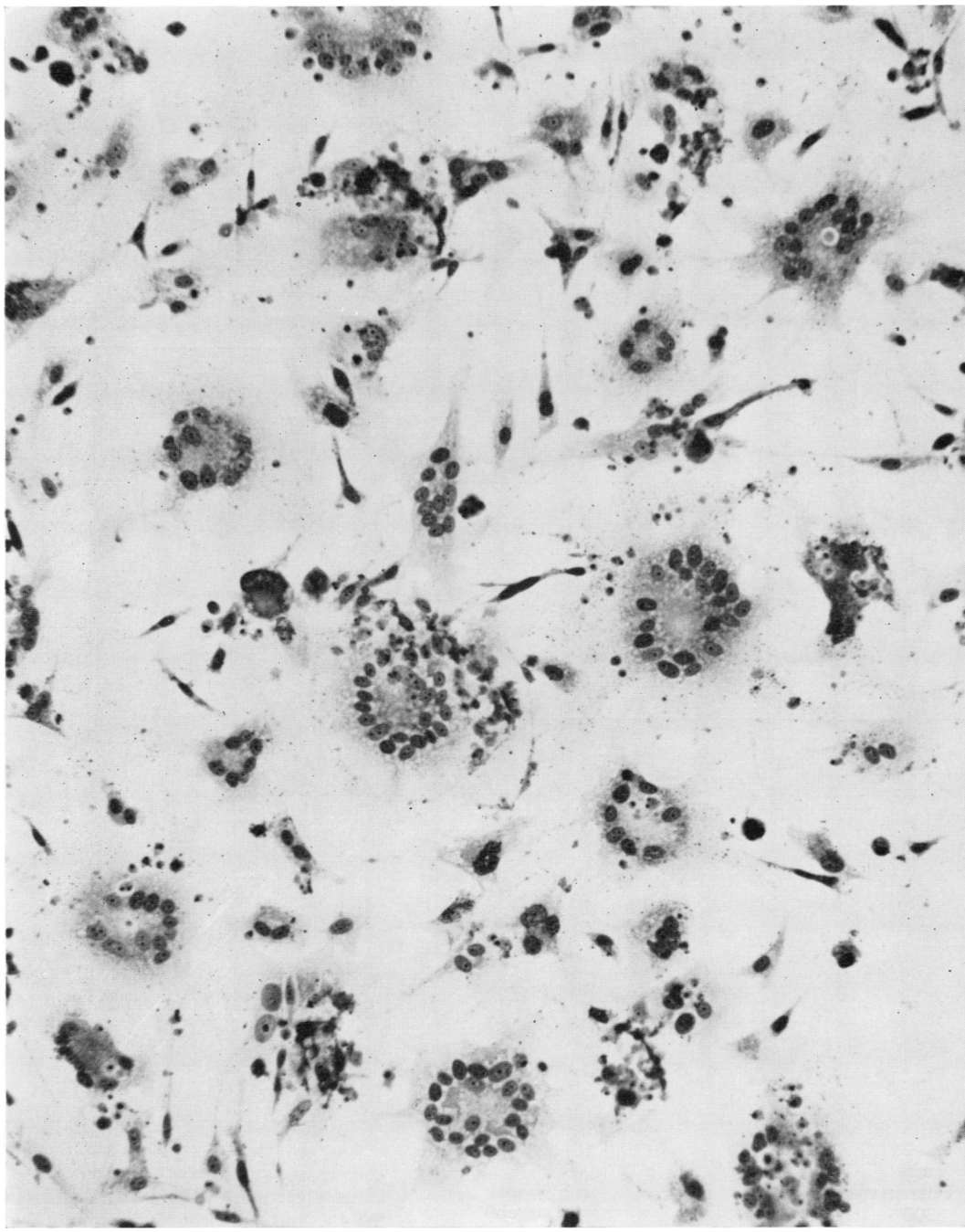

FIG. 2. *Cytopathic effect induced by virus isolate in a cell line derived from a canine fibrosarcoma 11 days after inoculation. Multinucleated giant cells subsequently lyse. May-Grunwald-Giemsa stain.* × *200.*

Intracisternal particles were found within the confines of rough endoplasmic reticular membranes of infected cells (Fig. 5). They appeared as naked electron-lucent spheres of which the approximate average diameter was 45 nm. Although the walls of the cisternae appeared distended, enveloping coats around the particles were not obvious.

Negative stains of partially purified concentrated virus revealed spherical particles with poorly defined surface spikes (Fig. 4B). The presence of the surface projections was compatible with the studded appearance of the external surface of the virion in ultrathin sections of infected cell cultures.

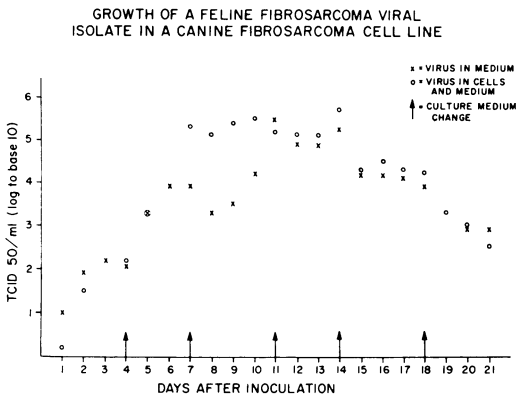

FIG. 3. *Biphasic growth curve of virus isolate in a cell line derived from a canine fibrosarcoma. Cultures inoculated with $10^{3.8}$ $TCID_{50}$/tube.*

TABLE 1. *Titer of virus isolate following chemical and physical exposures*

Type of exposure	Titer of exposed virus ($TCID_{50}$/ml)	Titer of sham-exposed virus ($TCID_{50}$/ml)
20% Ether, 18 hr, 4 C	No viable virus	$10^{5.2}$
4.8% Chloroform, 20 min, 4C	No viable virus	$10^{5.2}$
50 C, 30 min	$10^{2.2}$	$10^{5.2}$
37 C		$10^{5.5}$ (virus
24 hr	$10^{4.3}$	suspension)
3 days	$10^{3.2}$	
7 days	$10^{3.1}$	
4 C		
5 days	$10^{5.5}$	
114 days	$10^{3.9}$	
226 days	$10^{2.5}$	
376 days	No viable virus	
*p*H 3.05, 30 min, 4C	No viable virus	$10^{5.2}$ (*p*H 7.05)
Filtration (nm)		$10^{5.6}$ (un-
300	$10^{4.4}$	filtered)
220	$10^{3.3}$	
100	No viable virus	
50	No viable virus	

TABLE 2. *Response of virus isolate to 5-bromodeoxyuridine (BUDR)*

Treatment	Titer of virus isolate ($TCID_{50}$)	Titer of canine herpes virus ($TCID_{50}$)
Inoculum (0.2 ml/culture)	$10^{3.2}$/culture	$10^{4.9}$/culture
60 μg of BUDR		
6 days	$10^{4.2}$/ml	$10^{2.3}$/ml
10 days	$10^{5.2}$/ml	No viable virus
60 μg of BUDR + 60 μg of thymidine		
6 days	$10^{4.2}$/ml	$10^{3.5}$/ml
10 days	$10^{5.2}$/ml	$10^{3.5}$/ml
Control, no additives		
6 days	$10^{4.2}$/ml	$10^{4.3}$/ml
10 days	$10^{5.7}$/ml	$10^{3.3}$/ml

DISCUSSION

The demonstrated characteristics of the virus isolate make it the first of its type to be described for cats. Many of the characteristics are common to both the oncogenic RNA and myxovirus groups. Of the oncogenic feline viruses, three isolates induce leukemia and one induces fibrosarcomas in vivo (27, 28, 34, 38, 44, 46). All four have characteristics of the oncogenic type-C particles of the mouse and chicken and are presumably RNA viruses. Recognized myxoviruses of the cat have not been definitively identified to date (15, 20, 45).

Taxonomic criteria of the virus isolate which are common to both oncogenic RNA viruses and myxoviruses include RNA as nucleic acid type, sensitivity to lipolytics, acid lability, thermal lability, size, and insensitivity to antibiotics (23, 51, 53). A short communication of work done independently by others on the same tumor-tissue specimen from which the virus was isolated also reported sensitivity of the virus to ether and heat (33).

Some of the characteristics of development, size, and morphology of the virus isolate are also common to both the oncogenic RNA virus and myxovirus groups (17, 51). Development by a budding process from the plasma membrane of infected cells is common to both groups. The external diameter (106 nm) of the isolated virion falls within the ranges described for both types of virus. Structures comparable to the inner and intermediate membranes of feline, murine, and avian oncogenic virus particles were observed

(Fig. 4A; references 16, 24, 35, 37, 38, 44, 49, 50). Diametric dimensions of the particle enclosed within the membranes were closest to those reported by Laird et al. (37) and Dalton (16). In contrast, neither the external limiting membrane nor the electron-dense nucleoid of the classical type-C virion were demonstrable in observations of the isolated virus made to date (17). The sur-

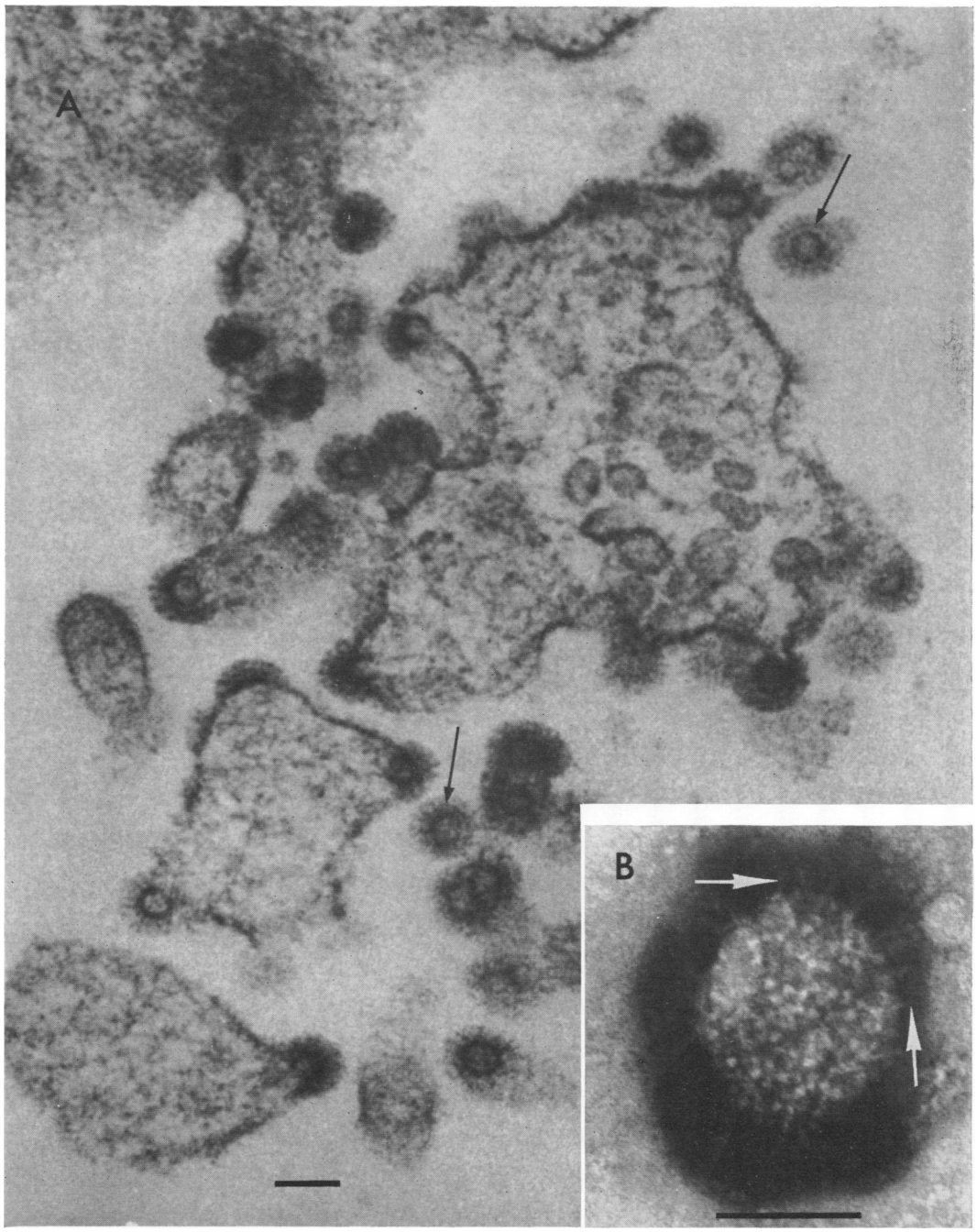

FIG. 4. *Virus isolate in various stages of budding from plasmalemma of infected indicator cell (A). Note membrane between nucleoid and external surface of some virions (arrows). Bar, 100 nm. Negative stain preparation (B) reveals poorly defined surface projections (arrows) from virion. Bar, 100 nm.*

face projections observed in negative stain preparations of the isolated virus (Fig. 4B) are characteristic for myxoviruses. They have also been noted in electron photomicrographs of the feline and avian leukemogenic viruses (5, 18, 38).

The significance of the intracisternal electron-lucent particles which were observed remains to be determined (Fig. 5). Intracisternal particles are also associated with murine leukemia (24) and feline infectious peritonitis (54), in which they

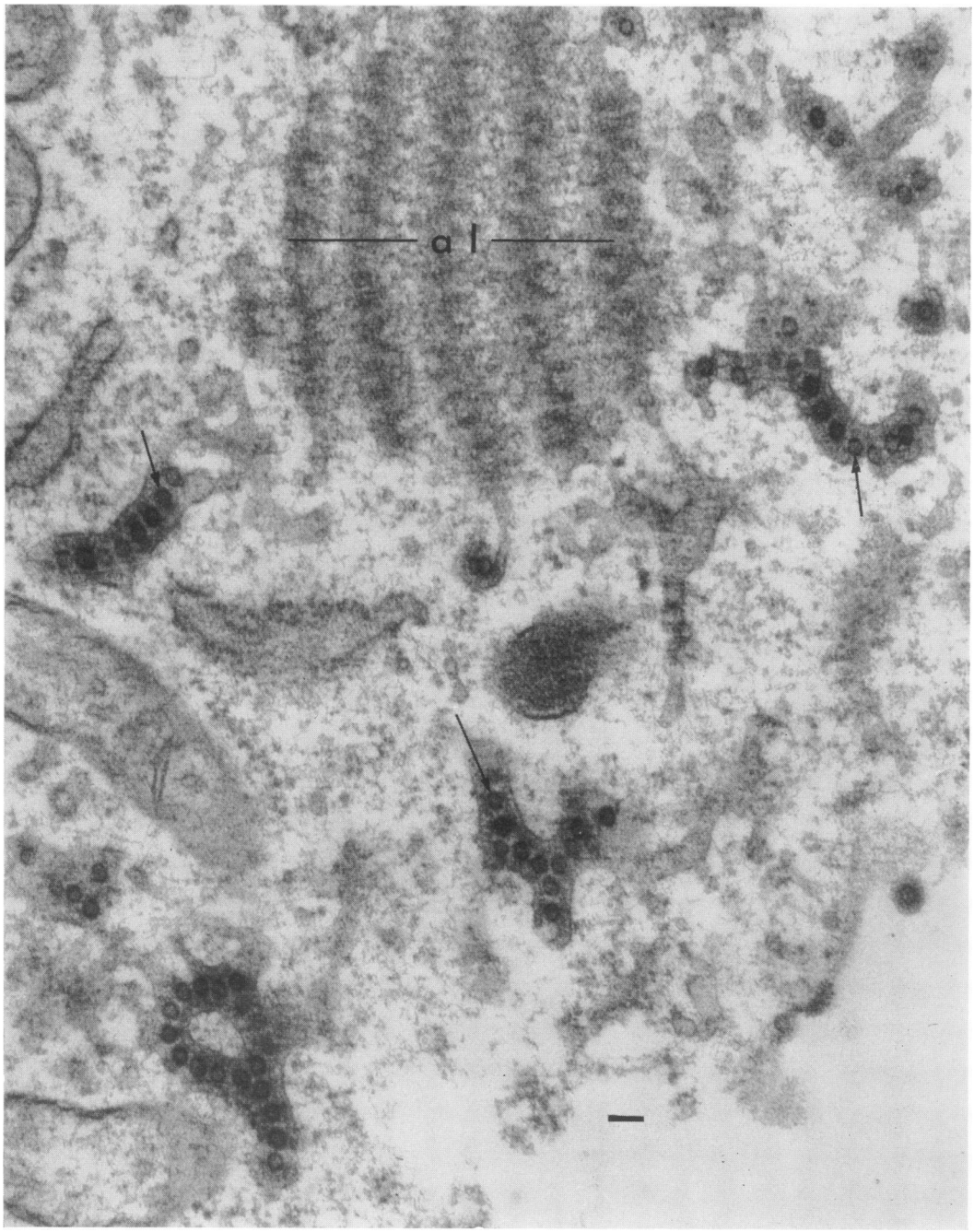

FIG. 5. *Naked intracisternal particles (arrows) within infected indicator cell. Annulate lamellae (al). Bar, 100 nm.*

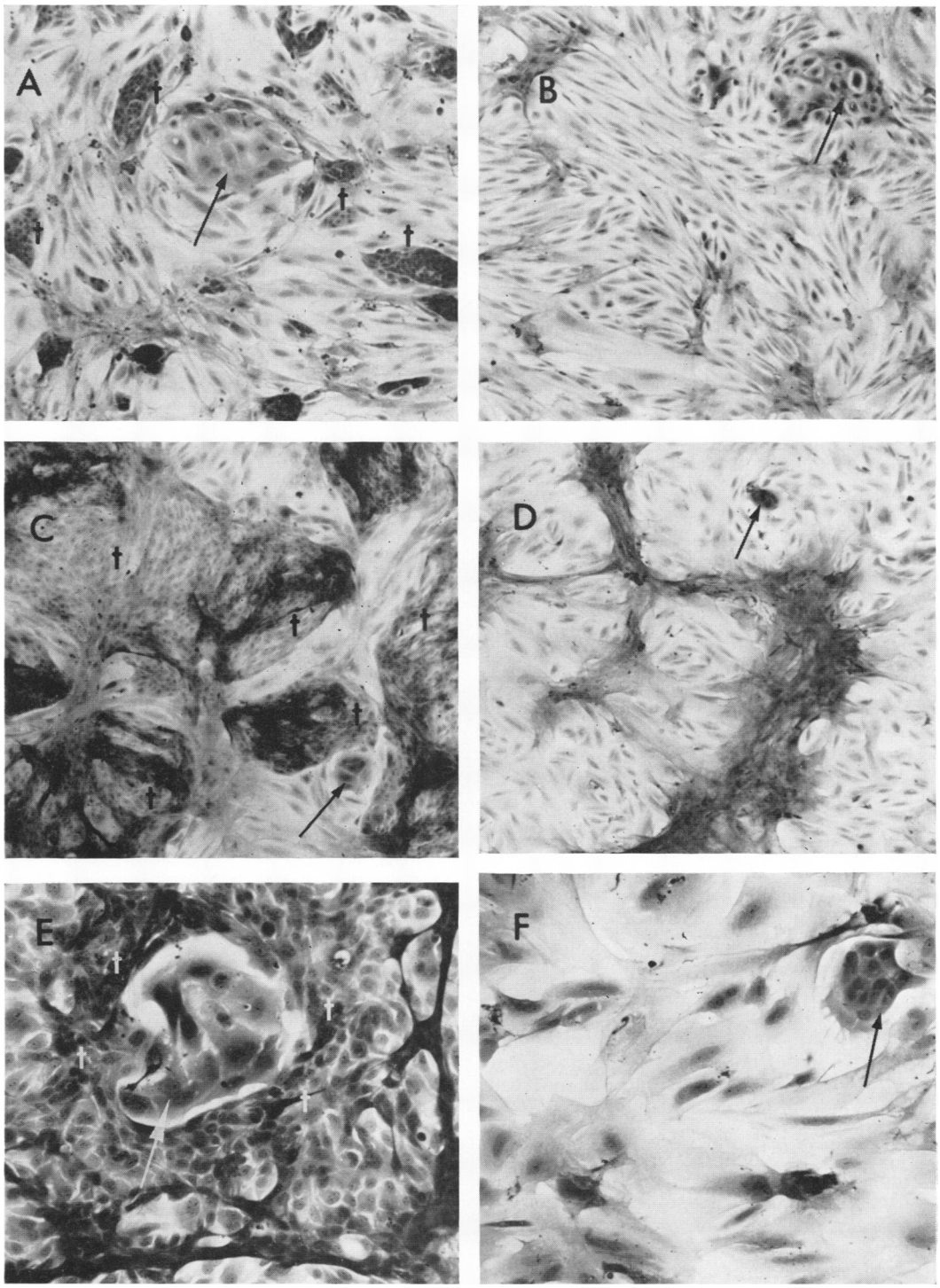

FIG. 6. *Nonlytic response of 6-day-old secondary feline renal cells to inoculation of virus isolate in left panels (A, C, E). Sham-inoculated chronological control cultures in right panels (B, D, F). Foci of normal renal epithelium indicated by arrows (A–F). Transformed foci (t) apparent on fourth postinoculation day (A) as compared to control culture of same age (B). Coalesced foci of transformed epithelium (t) overgrowing fibrocytes on postinoculation day 11 (C) as compared to ridges of fibrocytes in control cultures at same time (D). Focus of larger normal renal cells (arrow) with comparatively more cytoplasm completely surrounded by transformed cells (t) on postinoculation day 22 (E). Control cultures of same age with initial changes of senescence (F). Hematoxylin and eosin stain* $\times$ *50, A–D,* $\times$ *125 E and F.*

form by a budding process from rough endoplasmic reticular membranes. In contrast to the naked electron-lucent particles associated with the feline virus isolate, those associated with murine leukemia and feline infectious peritonitis have a wide electron-dense periphery or an enveloping membrane, or both. The significance of the intracisternal particles which are frequently seen in transplanted murine leukemic cells is also unknown at the present time (22, 24).

The CPE of multinucleated giant-cell formation and lysis is associated with myxovirus infections in contradistinction to the oncogenic RNA viruses. Recently, however, a virus with characteristics similar to the feline virus was isolated from tumor suspensions prepared from chickens with Marek's disease (8). The avian isolate was reported to produce both a syncytial CPE in vitro and neoplasms in chicks. Pathogenicity of the feline virus is yet to be determined. It has not caused clinical signs of disease to date after parenteral exposure of neonatal golden Syrian hamsters, weanling BALB/c mice, and limited numbers of neonatal kittens. No apparent signs of disease developed in 6-week-old dogs or adult rabbits after parenteral exposure to the virus by different routes. Thus the virus has not proven to be a virulent systemic pathogen in these species.

Inoculation of secondary feline renal cell cultures with the feline virus resulted in morphological transformation of the cells in two instances. Secondary Leighton-tube cultures were prepared from primary cultures of kidneys from 12 immature random-source cats. Cultures from 10 of the 12 animals responded to virus inoculation with a slow syncytial-lytic CPE and replication of virus to low titers. In contrast, all cultures from two of the cats (17 and 15 tubes, respectively) which were inoculated with the virus underwent a rapid epithelioid morphological transformation (Fig. 6). Both strains of transformed cells have undergone more than 45 subcultures in contrast to sham-inoculated control cultures which became senescent after 1 subculture. Cells or medium, or both, which were collected at numerous intervals from transformed and control cultures did not contain detectable virus when assayed as described for titration of the virus isolate.

A source of uniformly susceptible cells is critical to the demonstration of an unequivocal relationship between the virus isolate and cellular transformation. Inconsistency of the transforming response (16%) may have reflected the biological heterogeneity of the feline population which served as the source of cells. The influence of other factors to be considered include homogeneity of the virus pool, multiplicity of infection, chronological age of the cellular source, and kinetics of cultural growth.

The characteristics of the virus as demonstrated in these studies warrant further effort to definitively classify it. It is the first feline virus of this type to be described, and it appears to be either a form of a "defective" oncogenic RNA virus (25, 51, 52) or a pseudomyxovirus (53).

ACKNOWLEDGMENTS

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