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# STUDIES OF ENTERIC CORONAVIRUSES IN A FELINE CELL LINE

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## ABSTRACT

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Development is reported of a feline cell line which can support the growth of coronaviruses from canine (CCV), feline (FIPV) and porcine (TGEV) species. The cell culture has been serially transferred over 100 times and has retained its initial growth requirements, proliferative capacity and morphologic features. Each virus had specific growth characteristics in this cell culture although all produced a similar CPE and plaques under agar. Cross neutralization studies demonstrated a two-way relationship between TGEV and CCV and between TGEV and FIPV, whereas a one-way relationship was demonstrated between CCV and FIPV.

## INTRODUCTION

Coronaviruses have been associated with a wide variety of diseases in animals and human beings (Tyrrell et al., 1978). Investigations of the pathogenesis and basic properties of these viruses have been impeded due to the fastidious nature of their growth requirements (Robb and Bond, 1980). Cultivation of the enteric strains has usually been limited to their growth in the host animal or cells and tissues derived from that host (McIntosh, 1974). The feline infectious peritonitis virus (FIPV) has been grown at low infectivity titers by co-cultivation of infected peritoneal macrophages with feline cell cultures (Black, 1980; Pedersen et al., 1981a), in intestinal and tracheal ring cultures (Hoshino and Scott, 1978), in fetal feline lung cultures (O'Reilly et al., 1979; Hitchcock et al., 1981) and in the brains of newborn mice (Osterhaus et al., 1978a), rats and hamsters (Osterhaus et al., 1978b). The canine coronavirus (CCV) has been grown in canine kidney cell cultures (Binn et al., 1980) and in a human rectal adenocarcinoma cell line (Laporte and Bobulesco, 1981). The porcine transmissible gastroenteritis virus (TGEV) has been grown in primary and secondary pig kidney (McClurkin, 1965), thyroid (Tyrrell et al., 1978) and testes cultures (McClurkin and Norman, 1966), and has also been propagated in a primary canine kidney culture

(Welter, 1965). The absence of a single cell culture system capable of supporting the growth of coronaviruses from more than one animal species has limited studies of the antigenic and serological relationships among these viruses.

The purpose of the present report is to describe the development of a feline cell culture system that is capable of supporting the growth of canine, feline and porcine enteric coronaviruses. The viruses were adapted to the culture system so that cross-neutralization studies could be conducted among these viruses.

## MATERIALS AND METHODS

### *Fetal cat (FC) cell culture*

Primary cell cultures were prepared from the head and intestinal tract of 35-day-old fetuses obtained from a pathogen free cat. The fetuses ($n = 5$) were surgically removed from the uterus and placed in modified Eagle's minimum essential medium supplemented with lactalbumin hydrolysate (0.25%), sodium bicarbonate (26.1 m*M*), sodium pyruvate 2 m*M* and gentamicin sulfate (50 $\mu$g/ml). The medium was designated EMEM. The head and intestinal tract of the five fetuses that were removed were combined. They were washed 3 times in EMEM, minced into small pieces and trypsinized (0.25%) overnight at 4° C. The suspension was strained through gauze and the cells were sedimented by centrifugation at 600 *g* for 10 min. The sediment ed cells were washed 3 times in EMEM and resuspended at a concentration of $4.5 \times 10^5$ cells per ml in EMEM supplemented with 10% bovine fetal serum (BFS). Leighton culture tubes with 10.5 mm × 50 mm glass coverslips and 75 $cm^2$ plastic culture flasks (Falcon, Oxnard, CA) were seeded with 1.5 ml and 20.0 ml, respectively. Leighton tubes fitted with Morton closures and plastic flasks with loose caps were incubated at 37° C in a humidified atmosphere with 5% $CO_2$. Primary monolayer cultures were confluent by 14 days after seeding of tubes and flasks. Succeeding passages of FC cells were made at 3- to 7-day intervals at the same cell concentration.

### *Viruses*

Three different sources of FIPV were used: a liver suspension from an experimentally infected kitten designated UCD-1 AC/3 (Woods and Pedersen, 1979), a virus isolate grown in autochthonous cells (Pedersen, 1976) and homogenized liver samples from two cats with clinical feline infectious peritonitis.

A canine coronavirus, designated UCD-1 CCV, was obtained from Niels Pedersen, Davis, CA. This virus had been previously cultured in the A-72 canine cell line (Binn et al., 1980).

Two strains of virulent transmissible gastroenteritis virus, designated

Miller-3 (Frederick and Bohl, 1976) and SH-25 (Harada et al., 1967), were used. These viruses had been passaged 5 times in the McClurkin swine testes cell line (McClurkin and Norman, 1966).

*Virus growth*

Tenfold dilutions of the various virus suspensions were made in EMEM—2% BFS. The medium was aspirated from confluent FC cultures and each of three tubes or flasks were inoculated with 0.1 ml or 2.0 ml, respectively, of each virus dilution. After absorption for 1 h at 37° C, 1.5 ml or 20 ml, respectively, of conditioned media (media from 3- to 7-day-old uninfected FC cell cultures) was returned to the culture tubes or flasks. The infected cultures were incubated at 37° C and examined daily for CPE. When CPE was observed microscopically, the flasks were subpassed in a similar manner to normal cultures. During subpassage, it was found necessary to use about 10% of conditioned media for each new culture. Infected FIPV cultures were passed every 4 to 5 days to increase virus titer, as cell growth was usually in excess of cell destruction. The CCV and TGEV produced CPE on initial passage.

*Plaque assay*

Confluent monolayers of FC cells, grown in 60 × 15 plastic dishes (Falcon, Oxnard, CA), provided a suitable substrate for virus neutralization (VN) assays. Serial tenfold dilutions of 3× frozen-thawed virus-infected cell cultures were made in EMEM—2% BFS. The cell debris had been previously removed from the virus infected cultures by centrifugation at 10,000 *g* for 10 min at 4° C. The growth medium was aspirated from the cell cultures and 0.2 ml of each virus dilution was added to the plates. The plates were incubated for 1 h at 37° C and then overlaid with 5 ml of agar. The overlay consisted of equal parts of 2× EMEM 1% purified agar, 10% BFS and trypsin (20 μg/ml). Cultures were incubated for 2 to 6 days at 37° C for plaque development. After incubation, the agar was removed and the cells were fixed for 10 min with methyl alcohol and stained with 0.1% crystal violet. The virus titers were expressed as plaque forming units (pfu) per ml. All assays were run in duplicate cultures with three replicates run for each test.

*Antisera*

Feline anti-FIPV serum was obtained from specific-pathogen-free cats experimentally infected with the UCD-1 strain of FIPV. This serum was supplied by Niels Pedersen, Davis, CA.

Canine coronavirus antiserum was prepared in rabbits by the method of Reynolds et al. (1980). Blood for serum was obtained 14 days after the last inoculation.

Porcine antiserum to virulent TGEV strain Miller-3 was prepared in specific-pathogen-free pigs. Each pig was given orally 1000 pig infective doses of virus. Three weeks later the pigs were reexposed orally with another 1000 pig infective doses, followed by a third oral exposure 2 weeks later. Blood for serum was taken 10 days after the last exposure.

*Virus neutralization*

The VN activity was determined by plaque reduction test. All antisera were heated at 56° C for 30 min before use. Serial twofold dilutions were made in EMEM—2% BFS, mixed with an equal volume of virus suspension (75 pfu/0.1 ml) and incubated at 37° C for 1 h. Confluent FC culture dishes were inoculated with 0.2 ml of the serum-virus mixture and virus was absorbed for 1 h at 37° C. Cultures were overlaid with 5 ml of previously described agar overlay. Cultures inoculated with TGE or CCV virus required 2 days for plaques to develop, while those inoculated with FIPV required 5 to 6 days. The plates were fixed and stained as described. The highest dilution of serum showing 50% plaque reduction was considered the endpoint of the titration.

RESULTS

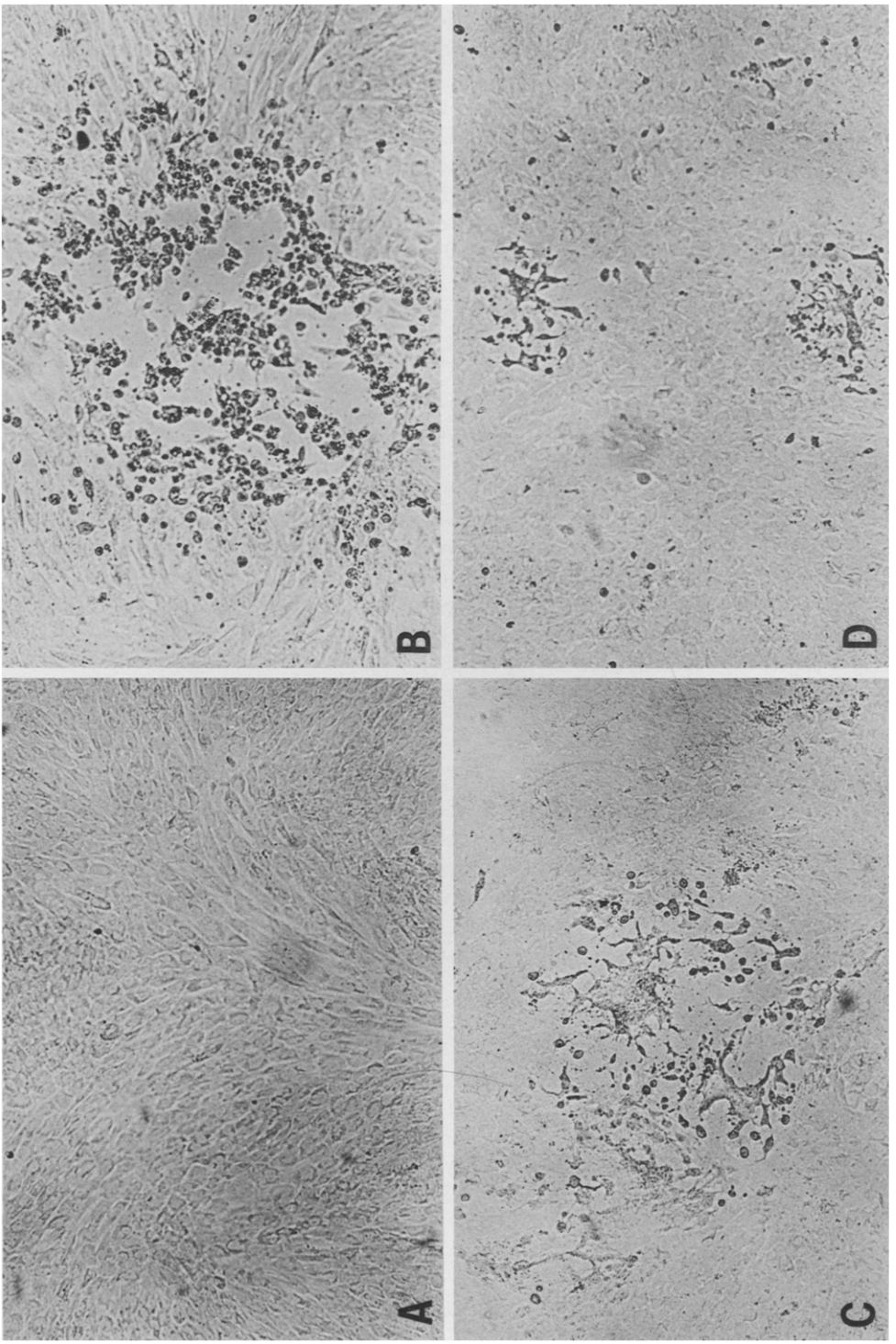
*Non-infected FC cell cultures*

Primary FC cell cultures were usually confluent by day 14. Microscopic examination revealed a monolayer of fibroblast-like cells (Fig. 1A). Once the cell culture was established, subcultures could be made every 3 to 7 days at a transfer ratio of 1 : 5. The FC cells have been maintained in culture for more than 100 transfers. During the prolonged serial passage (over an 18-month period), the morphological features of the cell culture have not changed. Tests to demonstrate viral and/or bacterial contamination of the cell culture have been negative.

*Growth of FIPV in FC cells*

Primary isolation of FIPV from infected tissues required at least three blind passages and frequently as many as five blind passages before virus growth and CPE was detected microscopically. The CPE was characterized by an increase in opacity of cells, rounding and detaching of cells, and occasionally, syncytium formation. The increase in opacity and rounding of

Fig. 1. Cytopathogenic effect of FIPV, CCV and TGEV infections on feline cultures at passage level 70 to 80. Unstained, × 175. (A) Uninfected FC cells in EMEM—10% BFS after 6 days incubation at 37° C. (B) FIPV infected cells 96 h after inoculation. (C) CCV infected FC cells 30 h after inoculation. (D) TGEV infected FC cells 36 h after inoculation.


A
B
C
D

infected cells made the CPE easy to detect against the fibroblast-like cells in the rest of the culture (Fig. 1B). The CPE was focal and did not appear to spread from the initial foci. However, trypsin dispersion of infected cultures did distribute the infected cells among the non-infected cells and this procedure increased the number of foci in the subculture. The highest FIPV titers were obtained when viral infected cell culture fluids were mixed with normal FC cells at the time plates and flasks were seeded and then allowed to become confluent. Viral plaque titers of $1 \times 10^5$ pfu/ml or greater were obtained by this method. Stock FIPV, maintained at −80° C, had to be passaged every 6 months to maintain high FIPV plaque titers as the virus titer decreased 2 $\log_{10}$ units during this storage interval.

*Growth of CCV in FC cells*

A CPE was observed 24 h after inoculation of 5-day-old FC cells with CCV. The CPE was recognized by enlarged rounded and/or amorphous cells, an increase in opacity of cells, and formation of syncytia (Fig. 1C). Ballooned cells were observed in some infected cultures. After initial appearance of CPE, the virus spread rapidly over the entire cell sheet. The virus was serially passaged over 50 times in the FC cell culture. Viral plaque titers greater than $1 \times 10^7$ pfu/ml were obtained from FC cultures 36 h after infection. The virus was maintained frozen at −80° C for periods up to 1 year with only a slight decrease in infectivity titer.

*Growth of TGEV in FC cells*

Propagation of the two virulent TGEV's in the FC cells required at least 1 or 2 blind passages before a CPE could be observed. The CPE was similar to that observed with CCV and FIPV (Fig. 1D). Subpassage of TGEV in the cell culture increased the plaque titer but did not cause any change in the plaque size. Viral titers of $1 \times 10^6$ pfu/ml or greater were obtained from cultures previously infected for 48 h. The viruses have been maintained at −80° C for 1 year with a decline in infectivity titer of 1 $\log_{10}$.

TABLE I

Cross neutralization titers with FIPV, CCV and TGEV

| Antisera against | Viral antigen | | |
|---|---|---|---|
| | FIPV | CCV | TGEV |
| FIPV | 256[a] | 28 | 4 |
| CCV | < 4 | 278 | 122 |
| TGEV | 8 | 400 | 900 |

[a]Reciprocal of dilution resulting in a plaque reduction of 50%.

*Cross-virus neutralization in FC cells*

Viral plaque reduction assays were conducted with these three viruses in FC cells. Homologous and heterologous neutralizing titers are presented in Table I. Neutralization of the homologous virus occurred at higher titers than those obtained with either of the heterologous viruses. Antisera against TGEV and FIPV neutralized all three viruses at different levels while the CCV antisera neutralized TGEV and CCV but did not completely neutralize the FIPV.

## DISCUSSION

A feline cell culture was established from fetal tissues and passaged over 100 times with little change in its maintenance requirements, proliferative capacity or morphological features. During the initial establishment of the culture, conditioned media (10%) from the preceding passage was added to the subculture media. This appeared to enhance the growth of the FC cells. After 20 to 30 passages, this practice was discontinued and the cultures would still become confluent but not as quickly. This observation suggested that conditioned media enhanced the growth of the FC cell but was not essential.

Development of a cell culture susceptible to infection with coronaviruses from three animal species provided an important research tool because of the difficulties associated with in vitro cultivation of coronaviruses (Robb and Bond, 1980). Although the growth procedures for each virus were different, they all produced a CPE in the FC cells that was similar to that reported for cultivation of each virus in cells or tissues derived for the host animal (Pedersen et al., 1981a; Binn et al., 1975; Kemeny, 1978). While the FC cell culture supported the growth of these viruses, it would be less useful for primary isolation of individual viruses from field cases because of initial low viral titers and the need for blind passages. Another disadvantage is the limited number of virus strains tested in the cells. There are numerous other strains of each virus that may or may not replicate in this system. The addition of trypsin to the agar overlay is a requirement for the formation of FIPV plaques, and it enhanced the development of CCV and TGEV plaques. The reasons for this were not investigated, although it is in agreement with a previous report on the use of trypsin with bovine coronavirus (Storz et al., 1981).

Results of limited cross neutralization assays demonstrated that serological relationships exist among these enteric coronaviruses. The antigenic relationships previously reported between TGEV and CCV, and TGEV and FIPV, were confirmed (Cartwright and Lucas, 1972; Pedersen et al., 1978; Reynolds et al., 1980). A strong two-way neutralization activity occurred between TGEV and CCV while a much weaker activity occurred between TGEV and FIPV. In contrast, the relationship between CCV and FIPV ap-

peared to be one-way, because FIPV antisera would neutralize CCV but CCV antisera would not neutralize FIPV. The differences reported in this study and those of previous reports may be due to the low number of samples evaluated in this study. However, since all three viruses replicate in this system, the exact interrelationships among these viruses may now be much easier to determine. In addition, the relationship of the recently reported feline enteric coronavirus (Pedersen et al., 1981b) to these viruses will be much easier to evaluate.

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