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DEVELOPMENT, CHARACTERIZATION, AND VIRAL SUSCEPTIBILITY OF A FELINE (*FELIS CATUS*) RENAL CELL LINE (CRFK)*

ROBERT A. CRANDELL, CATHERINE G. FABRICANT AND WALTER A. NELSON-REES

Laboratories of Veterinary Diagnostic Medicine, University of Illinois, Urbana, Illinois 61801; Department of Microbiology, New York State Veterinary College, Cornell University, Ithaca, New York 14850; Naval Biomedical Research Laboratory, School of Public Health, University of California, Oakland, California 94625

SUMMARY

Cell line CRFK, derived from kidney tissue of a normal domestic kitten, was initiated in 1964. With intermittent periods of storage in the frozen state, it has been grown in vitro during more than 200 passages, without apparent loss of susceptibility to selected viruses. Various herpesviruses and feline viruses belonging to different virus groups grow readily and with distinct cytopathic features. The cells now grow as a smooth monolayer of epithelial-like cells; most have 37 chromosomes (2n-1) and are thus aneuploid for cat karyotype. Three distinct marker chromosomes are identified. The cell line, which is free of mycoplasmal contamination, is useful in feline virus research and diagnostic medicine and has become of particular interest in cancer research.

Early in the period of the feline virus investigations, the need for a feline cell line was recognized. Since the feline viruses under study at the time propagated only in cells of feline origin, the constant requirement for primary feline cell cultures was a hindrance to their research. The development of a cell line was also prompted by the detection and isolation of endogenous agents in primary cell cultures. An attempt to establish a feline cell line was initiated by one of us (R. A. C.) in the United States Air Force Epidemiological Laboratory, Lackland Air Force Base, Texas, in June 1964. The resultant cell line, referred to in literature as the CRFK (1–4) and CCC (5–7) feline kidney cell line, has been cultured or preserved at various passage levels since that time. It is unique in the sense that other investigators have failed to maintain feline renal cells in continuous culture. We propose the continued use of the designation CRFK for Crandell feline kidney cell exclusively. CRFK cells have been employed in feline virus research in a variety of ways. They served to isolate a feline syncytia-forming virus from cats with experimentally induced or spontaneous urolithiasis (1, 8). They were found to be susceptible to eight different feline picornaviruses (caliciviruses) including the Manx picornaviruses (9, 10); they were susceptible to the feline panleucopenia virus (4) and a variant of bovine parainfluenza-3 replicated with syncytia formation (11). CRFK cells were more uniformly susceptible to two strains of feline reovirus, panleucopenia, and to a new feline herpesvirus serotype (12) than many primary feline cultures. Feline herpesvirus was shown to induce the formation of mineral crystals in CRFK cells (13), and a feline leukemia pseudotype of murine sarcoma virus, MS (FeLV), induced focus formation in CRFK monolayers (5).

Lack of oncogenicity was demonstrated by the

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failure of the CRFK cells to induce tumor formation in the Syrian hamster cheek pouch (*unpublished results*, 1971). No extraneous viruses and C-type particles were found by electron microscopy, complement fixation, and immunofluorescence (5), nor did labeling with [^{3}H]uridine show a radioactive (RNA) banding pattern characteristic of RNA C-type viruses following isopycnic centrifugation (A. J. Hackett, *unpublished results*, 1971). However, more recently Livingston and Todaro (6), Sarma et al. (7), Okabe et al. (14), and Fischinger et al. (15) have found, in CRFK cells from different sources, a C-type virus that has antigenic properties related to those of the RD-114 virus (16, 17).

The purposes of this paper are to record the history of the CRFK line during four phases of development, and to describe certain of its culture characteristics and its susceptibility to other selected viruses.

Materials and Methods

Cell source and cultivation. CRFK cells were derived from the cortical portion of the kidneys of a 10- to 12-week-old normal female domestic cat (*Felis catus*). Primary cultures were prepared by the trypsin digestion method of Madin et al. (18). The growth medium was Hanks' balanced salt solution supplemented with essential amino acids, vitamins, L-glutamine, and 10% calf serum. All media contained 200 I.U. of penicillin, 100 μg of streptomycin and 20 I.U. of amphotericin B (Fungizone) per ml. The pH was adjusted to 7.2 to 7.4 with $NaHCO_3$, and the cells were grown as stationary cultures in stoppered tubes and bottles at 35 to 36°C.

Monitoring for microbial contaminants: mycoplasmas. During phase 4 (C.G.F.) all cell lots (controls, stocks for freezing or distributing) and media, sera, trypsin, etc. were constantly monitored for mycoplasmal contamination on at least five or more media as described by Fabricant and Barber (19) and Al-Auibadi and Fabricant (20). The liquid and corresponding agar media were inoculated with portions of cell culture fluids (sera, trypsin, etc.) and incubated at 35°C for 6 days. The agar plates were placed in candle jars with excess moisture prior to incubation (21). If the agar media were negative after the incubation, the broth media were plated onto fresh corresponding solid media and incubated as described above. The cells (or media ingredients) were considered free of mycoplasmas if all cultures were negative.

Other microbial agents. Lots of growth, maintenance, and freezing media and medium ingredients were incubated in 5-ml quantities; smaller samples were inoculated in thioglycollate, Sabouraud dextrose, and Sabouraud maltose agar, and were incubated at 35°C. Duplicate cultures were made on the latter two mycotic media and incubated at 25°C.

Freezing and storage of cell stocks. Cell cultures 24 to 48 hr old were used as freezing stock. The cells were treated as described for subculturing the fourth phase of cell culture. The cells were resuspended in growth medium containing 20% serum and 5% dimethylsulfoxide (Fisher D-136 certified and spectroanalyzed). The number of cells dispensed in 1 ml of medium per sterile freezing ampule (Wheaton) was approximately the number of cells growing confluently on a surface of 15 cm^2. The time for which the cells remained in contact with the dimethylsulfoxide prior to freezing was found to be critical. The best cell viability was obtained when the exposure was reduced to a minimum. The ampules were sealed quickly with an oxygen-natural gas flame, placed in a Virtis controlled freezer for 30 min, and then stored in liquid nitrogen.

Chromosome observations. Cells were prepared for karyology by the air drying method (22) and stained with Giemsa. The karyotype was compared to that reported by Hsu and Benirschke (23).

Viruses and clinical specimens. The viruses employed in this study were maintained as stock cultures in the United States Air Force Epidemiological Laboratory, and their identity was confirmed by serum neutralization tests. Clinical specimens consisted of nasal and oropharyngeal swabs obtained from cattle and kittens, respectively. Tissue suspensions of brains from pigs naturally infected with pseudorabies virus were prepared by standard methods. Each of four culture tubes was inoculated with 0.1 ml of undiluted and 0.1 ml of a 1:1000 dilution of each virus. Clinical specimens were inoculated in 0.1- to 0.2-ml amounts into each of four culture tubes. When 50 to 60% of the cells showed evidence of cytopathic effect (CPE), 0.1 ml each

of fluid and cells was passed again, undiluted and at 1:1000 dilution.

Virus titration. Serial 10-fold dilutions of cell culture fluid containing virus were prepared, using nutrient fluid as the diluent. Of each dilution, 0.1 ml was inoculated into each of the four cell culture tubes. The end point was calculated (24) and expressed as the 50% tissure culture infective dose ($TCID_{50}$) per 0.1 ml of inoculum.

Histologic preparations. Coverslips were stained with either May-Grünwald-Giemsa after alcohol fixation or hematoxylin and eosin following fixation in Bouin's fluid.

Results

Phase 1, establishment. The initiation, cultivation, and derivation of the stock of *Mycoplasma*-free CRFK cell line can be divided into four phases. (*1*) Establishment and growth to 84th passage at Lackland Air Force Base by R. A. C. (*2*) Growth to 132nd passage at Pitman-Moore Co. by Dr. J. Bittle. (*3*) Growth to 146th passage at Cornell University by Dr. K. M. Lee. (*4*) Derivation of mycoplasma-free stock and growth to present high passage CRFK at Cornell University by C. G. F. The primary monolayer was confluent after 5 days incubation and consisted primarily of epithelial-like cells. The growth medium was removed, a 0.25% trypsin (1:250) solution added, and the culture held at room temperature for 10 min. The trypsin solution was removed after slow speed centrifugation and the cells again dispersed by pipetting back and forth in fresh growth medium (Table 1). During much of this time the cells were passed at a low ratio with a 2-fold increase in 7 to 10 days.

Phase 2, cultivation. In Dr. Bittle's laboratory the culture medium was changed to Eagle's minium essential medium (MEM) with nonessential amino acids and 10% fetal calf serum. The serum was reduced to 2% for the maintenance medium (Table 1).

Phase 3, cultivation. At Dr. Lee's laboratory (Cornell), the presence of mycoplasmas was recorded in the second subculture after receipt. Treatment with tetracycline and GIBCO anti-PPLO agent was unsuccessful in eliminating the mycoplasmas (Table 2).

A lot of cells was frozen and stored in liquid nitrogen at the 146th passage on July 6, 1967. The age of these cells and the composition of the freezing medium are unknown.

After thawing and subculturing 16 times, these cells also were found to be contaminated with mycoplasmas on October 6, 1967.

Phase 4, cultivations and the derivation of the Mycoplasma-free stock. One vial of the lot of cells mentioned above (representing the 146th passage) was given to one of us (C. G. F.) approximately 5 months after freezing. These cells were thawed quickly in cold tap water. The

TABLE 1

First and Second Phase Histories of CRFK Cell Line Development

Passage No.	Date of Passage	Treatment	Date and Result of *Mycoplasma* Treatment
First phase*			
Primary	June 1964	None	July 1965; mycoplasmas isolated†
Passaged every 5 to 10 days	July 1965	None	
Three successive passages	July 1965	Kantrex (Bristol) 200 mg/ml	Unknown
84‡	January 1966		
190	June 1967	(All cultures lost due to mechanical failure)	
Second phase§			
84	January 1966	None	
132¶	March 1967	None	

* R.A.C., United States Air Force Epidemiological Laboratory, Lackland Air Force Base, Texas.

† By agar and broth culture methods (courtesy of R. B. Owens, Naval Biomedical Research Laboratory, Oakland, Calif.).

‡ A sample culture, passage 84, sent to Dr. J. Bittle in January 1966 (second phase).

§ J. Bittle, Pitman-Moore Co.

¶ A sample culture, passage 132, sent to Dr. K. M. Lee, Department of Microbiology, Cornell University.

TABLE 2

THIRD PHASE HISTORY OF PASSAGE, ANTIBIOTIC TREATMENT, AND RESULTS OF *MYCOPLASMA* TESTING OF CRFK AFTER PASSAGE 132

Passage No.	Date of Passage	Treatment	Date and Result of Test for *Mycoplasma*
134	3/13/67	None	3/24/67 +*
137a	3/30/67	Tetracycline 10 μg/ml	4/5/67 +
137b	3/30/67	Tetracycline 50 μg/ml	4/5/67 +
138	4/14/67	Tetracycline 10 μg/ml	4/18/67 +
142	4/2/67	Tetracycline 50 μg/ml†	4/9/67 +
146‡	Not known	7/21/67 GIBCO anti-PPLO added	4/28/67 +
162§	9/28/67	None	10/6/67 +

* Mycoplasmas isolated.

† Level and agent not recorded, but probably as indicated.

‡ This passage frozen. One vial subsequently found to be *Mycoplasma*-free and gives rise to phase 4 (Table 3).

§ Another vial of passage 146 thawed and passaged 16 times.

content of the vial was withdrawn and resuspended in 40 to 50 ml of MEM with Earle's salts, 10% fetal calf serum, and 0.5% lactalbumin hydrolysate, adjusted to pH 7.3 to 7.4 with 7.5% $NaHCO_3$.

The fluid was changed to a maintenance medium (same as the growth medium except that the fetal calf serum was reduced to 1%) when the cells were confluent (1 to 3 days).

The second passage (148th) after thawing was found to be free of mycoplasmas. Three flasks of the fifth passage (151st) and two flasks of the sixth passage (152nd) of these cells were also negative for mycoplasmas (Table 3). The *Mycoplasma*-free stock of cells was frozen and represented a total of 151 passages. Distribution of these cells (C. G. F.) began within a few months after preparation of the stock.

For subculturing, the medium is decanted and the cells are washed with sterile phosphate-buffered saline (PBS) and treated with a mixture of 0.05% trypsin and 0.05% Versene for 30 sec. This mixture is decanted and the cells incubated in an inverted position for 10 min at 35°C. The flask is tapped gently to loosen the cells, and the cells are resuspended in 5 ml of growth medium with the aid of a pipette and sterile rubber pipetting bulb. No oral pipetting was permitted in media or cell preparation, to avoid mycoplasmal contamination of cell cultures from respiratory droplets. No other cell types were handled at the time CRFK stocks were being prepared.

The pH and the depth of the culture medium influenced the cell growth. The cells grew and were maintained well at a pH between 6.8 to 7.4. Cells maintained poorly if more than 40 to 50 ml of medium were added per 250-ml disposable plastic flask. Perhaps this was due to an interference in the gaseous exchange between the medium and atmosphere. Various environmental factors were found to influence the viability of the cells (Fabricant 1969–1973, *unpublished data*). Rubber stoppers and glass culture flasks which had been repeatedly used were often toxic to the cells. Certain lots of disposable culture flasks were toxic, and some were contaminated.

When cultured, the *Mycoplasma*-free cells maintained a uniform growth pattern and attained confluency in 1 to 3 days. In the unstained preparations and in preparations stained with a modified May-Grünwald-Giemsa, the cells appeared epithelial-like and the cytoplasm was not granular or vacuolated. Representative cell cultures have to date undergone over 200 serial transfers. The cells have retained their epithelial-like morphology (Fig. 1), and cultures

TABLE 3

FOURTH PHASE HISTORY OF PASSAGE AND DERIVATION OF *MYCOPLASMA*-FREE STOCK FROM ONE FROZEN VIAL, PASSAGE 146, OF PHASE 3

Passage No.	Date of Passage	Treatment	Date and Result of Test for *Mycoplasma* (Control No.)
146	1/5/68	None	Not done
147	1/7/68	None	Not done
148	1/15/68	None	1/18/68*
151	2/23/68	None	2/24/68—(713) —(714) —(715)
152	2/23/68	None	2/24/68—(718) —(719)
154†	6/7/68	None	6/7/68—(917)

* No mycoplasmas isolated.

† A vial of cells frozen at passage 151 with a control number was thawed and cultured for three passages, and a new lot frozen.

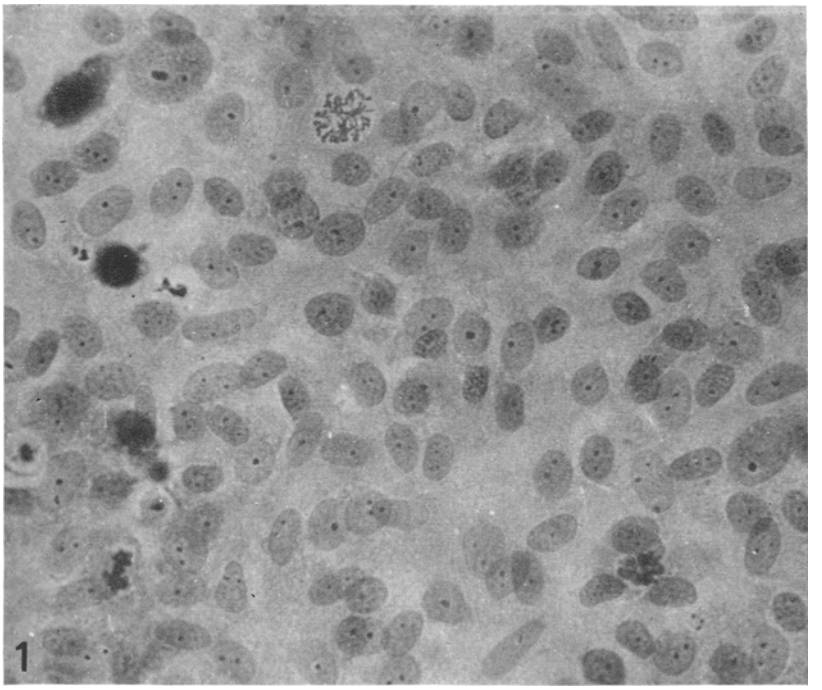

FIG. 1. Monolayer of *Mycoplasma*-free (phase 4) CRFK cells, passage 160. May-Grünwald. Leighton coverglass. × 530.

can be split at a ratio of 1:5 or more depending upon the amount of growth at the time of transfer.

Except for a brief statement (5) to the effect that a strain (CCC) of the CRFK line, frequently cloned, "was aneuploid for cat karyotype," we know of no published record regarding its chromosome constitution.

One of us (W. A. N-R.) first observed the chromosomes in cultured cells at passage 74 (received from R. A. C. at passage 73) in December 1965 and subsequently at passages 85 and 89 in March 1966. Over 50% of the metaphases in these passages contained chromosome and chromatid fragments. There was an abundance of dicentric chromosomes and concomitant appearance of chromatin bridges in bi- and multinucleated cells, resembling a similar situation then being observed in a bovine testicular cell line (25). Polyploidy appeared in 20 to 25% of the cells. The modal number of chromosomes fluctuated between 40 and 47 in five different preparations (passages 74 to 89), but was deemed unreliable because of the high incidence of aberrations and polyploidy.

A somewhat more stable population of cells was observed in May 1967 at passage 187 (also received from R. A. C.). Eight of 24 cells had 42 chromosomes, and although 11 of 24 bore aberrations, dicentrics were fewer (2 of 24), polyploidy was 8.3%, and there were very few multinucleated cells.

In addition to other aberrant chromosomes, some metaphases bore three distinct markers: (*1*) the normal E_1 chromosomes (reference 23); (*2*) a chromosome similar to E_1 bearing a pronounced satellite, but with longer long arms, approximately doubling the total length of the chromosome; (*3*) a single long subtelocentric chromosome (Fig. 2).

All of these cultures were from phase 1 and were found to be contaminated with mycoplasmas.

In January 1971, a culture of cells bearing the description CRFK, which had been passaged a total of 154 times, was received (from the laboratory of C.G.F., i.e. phase 4). This culture was free of mycoplasmas.† Morphologically and

† By electron microscopy and uridine labeling (by courtesy of A. J. Hackett) or by agar and broth culture methods (by courtesy of L. Hayflick).

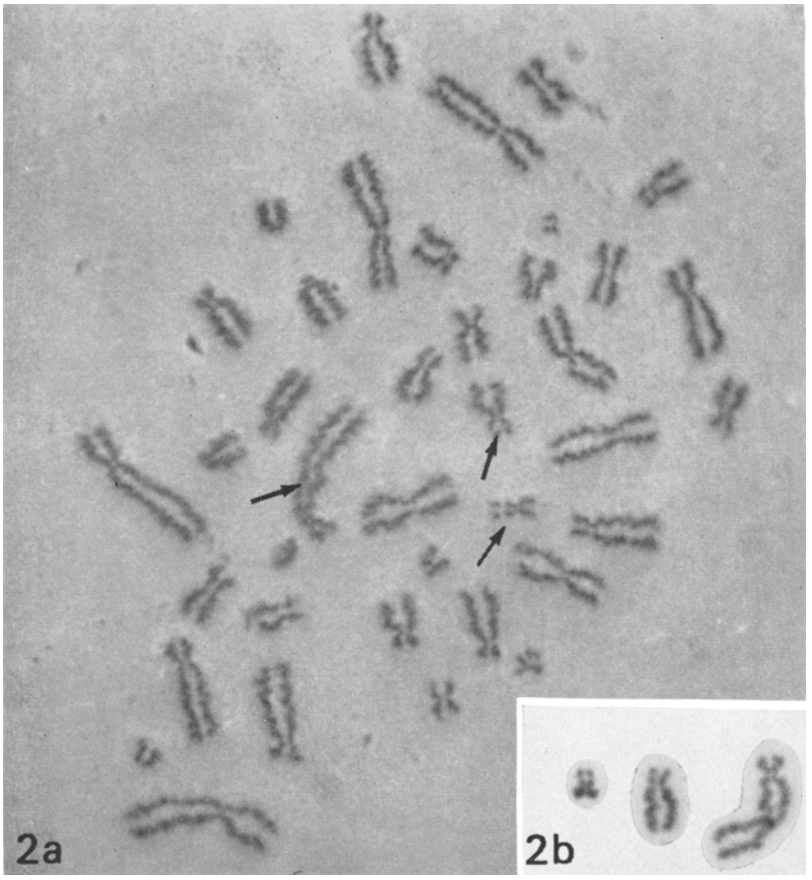

FIG. 2. *a*, metaphase of high passage (phase 1) culture of CRFK line, prior to mycoplasmal decontamination. Three marker chromosomes (*arrows*). *b* (*inset*), three marker chromosomes from another metaphase.

chromosomally it reflected a very uniform population. Table 4 indicates that the majority of cells had 37 chromosomes (2n-1), thus aneuploid for cat karyotype (23), and 90% were characterized by the presence of marker chromosomes the same as or similar to those described above.

This culture had not been purposely cloned but represents the survivors of various cultural procedures, including drug treatment for mycoplasmal contamination and freezing. Fig. 3 shows idiograms of four CRFK metaphases with 36 to 38 chromosomes, each marked at least by the peculiar satellited chromosome, a long subtelocentric chromosome, and one regular E_1 chromosome.

Virus susceptibility. The six herpesviruses and eight feline picornaviruses (caliciviruses) listed in Table 5 replicated in cultures of CRFK cells with demonstrable cytopathic effect (CPE). The cytopathogenicity of all viruses was very distinct. In stained preparations intranuclear inclusion bodies were demonstrated in cultures

TABLE 4

CHROMOSOME DISTRIBUTIONS IN 23 CRFK CELLS OF *MYCOPLASMA*-FREE CULTURE, PASSAGE 167 (Phase 4)

No. of Chromosomes	No. of Cells*
35	1
36	7
37	13
38	1
39	1

* All cells except two with 36 chromosomes and one with 38 bore similar chromosome markers (see Fig. 3).

FIG. 3. Idiograms of four CRFK metaphases of *Mycoplasma*-free cells (phase 4), passage 167, with 36 to 38 chromosomes. All cells bear at least three marker chromosomes (*arrows*) similar to those in Fig. 2, *a* and *b*.

TABLE 5

SUSCEPTIBILITY OF CRFK CULTURES TO SELECTED HERPESVIRUSES AND FELINE PICORNAVIRUSES

Virus	Titer, Log $TCID_{50}$ per 0.1 ml
Pseudorabies	7.0
Herpes simplex	6.6
Equine rhinopneumonitis	6.0
Monkey B	5.0
Infectious bovine rhinotracheitis	6.5
Feline viral rhinotracheitis	6.6
Feline respiratory isolate—6	8.3
Feline respiratory isolate—12	8.3
Feline respiratory isolate—14	8.5
Feline respiratory isolate—29	7.0
Feline respiratory isolate—278	8.3
California feline isolate (CFI)	7.3
Kidney cell degenerating (KCD)	7.0
FPL (Bolin)	7.0

infected with the viruses of pseudorabies, herpes simplex, equine rhinopneumonitis, monkey B, infectious bovine rhinotracheitis, and feline rhinotracheitis. In each instance, the virus assayed for infectivity represented one to a million or greater dilution of the original virus inoculum. The resultant titers were similar to those obtained with the same viruses propagated in cultures of primary feline renal cells (PFK). Although the virus titers shown in Table 5 were obtained at various passage levels (11 to 164) during the first phase of cultivation, similar titers were obtained when many of the viruses were tested in cells derived from the fourth phase.

We compared the sensitivity of both the CRFK and PFK cultures to these viruses and to primary virus isolations from cats. All viruses used in the comparative infectivity tests had been passaged in primary feline cells between seven and 13 times. The results of the comparative titrations are presented in Table 6 and indicate that the CRFK cells and the PFK cells were equally sensitive to these viruses.

Viruses (one herpesvirus and eight picornaviruses) were isolated from the same nine kittens in culture systems of both cells. Distinct and similar CPE occurred in both cell systems. These results were duplicated using a higher passage of the CRFK cells derived from both the first and fourth phases of cultivation. The viruses of infectious bovine rhinotracheitis and pseudorabies were isolated in CRFK cultures inoculated with nasal swabs from cattle and brain tissue suspensions from infected pigs.

DISCUSSION

The morphology of the cells throughout the development of the line has been epithelial-like. Two hundred passages have been achieved over a 9-year period with intermittent storage in the frozen state. Although their initial growth rate was slow, the cells now propagate sufficiently to provide adequate amounts of cultures for laboratory and commercial use.

In two separate laboratories a rapid increase in growth was recorded after the 90th passage, or approximately 2 years of continuous cultivation.

Chromosomally, the cells of the present *Mycoplasma*-free CRFK line represent a rather uniform population as compared to observations on other strains and passage levels. Three marker chromosomes observed in some cells of earlier cultures of higher passage levels and among a very heterogenous array of *Mycoplasma*-contaminated cells are now found in most cells of the present population and among cells with a narrow range of chromosome numbers. We speculate that the present CRFK line, which represents survivors of drug treatment and a frozen-preserved sample of the previously contaminated culture, is de facto a clone or derivative of one or a few chromosomally similar cells—in essence, conforming to a similar pattern of events described by Stanbridge (26) where treatment for mycoplasmal infection may have led to selection of a pre-existing population and thus of a specific marker(s) chromosome.

The *Mycoplasma* control measures utilized in

TABLE 6

COMPARATIVE INFECTIVITY TITERS OF SELECTED VIRUSES ON CULTURES OF PRIMARY FELINE RENAL CELLS (PFK) AND CRFK

Virus	Titer Log $TCID_{50}$ per 0.1 ml	
	PFK	CRFK
Pseudorabies	6.5	6.6
Herpes simplex	6.0	6.0
Monkey B	5.0	5.0
Infectious bovine rhinotracheitis	5.5	6.0
Feline viral rhinotracheitis	6.6	6.5
Feline respiratory isolate—12	7.6	7.5
Feline respiratory isolate—6	8.3	8.0

phase 4 of this study were considered imperative because of the numerous potential sources of mycoplasmas and because former passage levels were found to be contaminated. Mycoplasmal contaminants may come from careless techniques (via respiratory droplets, by mouth pipetting, hands), various media components (sera, trypsin, etc.), and by the nature of the laboratory work itself (27). *Mycoplasma* species from these heterogeneous sources vary considerably in their cultural requirements, ranging from extremely fastidious to relatively simple (21, 28). Because of this wide difference in cultural characteristics, dependence upon one medium to monitor successfully for mycoplasmal contamination is unreliable.

Some investigators have shown that single antibiotic treatment of infected cells is not always effective (29, 30). Attempts to rid other cell cultures of mycoplasmal contamination by antibiotic treatment, including kanamycin, have failed (C. G. Fabricant, *unpublished data* 1968–1970, and 24). These treatments were found to be toxic to the cells even though mycoplasmas survived. Antibiotics effective against pure cultures of mycoplasmas have seldom been effective in "curing" all cultures of mycoplasmal contamination. (C. G. Fabricant, *unpublished results*, 1968–1970, and 31). Because of these experiences, we speculate that perhaps the one vial found to be free of mycoplasmas in this study was due to failure of the mycoplasmas to survive the low temperature during the 5 months storage. Some mycoplasmas do not survive under these conditions (C. G. Fabricant, *unpublished data* 1960–1970, and 31). Since this *Mycoplasma*-free stock was established, mycoplasmas have not been demonstrated by electron microscopy or by cultural methods.

The virus susceptibility of CRFK cells is very similar to that of primary feline renal cells, and CPE of the herpesviruses and picornaviruses is like that previously reported for these viruses in primary feline cell cultures (32, 33). The continued monitoring of the cells for sensitivity to virus infection revealed no detectable change with long term cultivation of the cells.

The CRFK cell line may provide a tool for studying the release mechanism of viruses from feline cells after long term cultivation as has been reported for murine (34) and avian cells (35).

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