Mammalian Cells in Culture Frequently Release Type C Viruses

Abstract. *Cell cultures commonly used in animal cell research, both cell strains and continuous cell lines from various mammalian species, spontaneously produce type C RNA viruses.*

We wish to report that many commonly used mammalian cell cultures from various species are producing readily detectable amounts of type C RNA viruses with biochemical and immunologic properties similar to those of known leukemia and sarcoma producing viruses (*1*). Virus production occurs from cell strains and continuous cell lines of different morphologic types and degrees of differentiation. Such virus release can begin spontaneously after hundreds of cell generations in vitro (*2*); it has not resulted in cytopathic alterations in those cells producing virus. To what extent the release of type C viruses by mammalian cell cultures may affect the results of experiments or constitute a significant biohazard to laboratory personnel remains to be determined.

Thirty-one cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland. Most of the nonprimate mammalian cell lines available were examined, excluding those of mouse origin which are already known to have a high probability of releasing endogenous type C virus (*2, 3*). Nine fetal feline cell strains were obtained from the Cell Culture Laboratory, Naval Biomedical Research Laboratory (NBRL), Oakland, California. The cells were received frozen in sealed vials; the vials were thawed and the contents were placed in plastic tissue culture flasks containing Dulbecco's modification of Eagle's minimal essential medium and 10 percent calf serum (Colorado Serum Co., Denver). The rat kidney cell line, NRK (*4*), and a subclone of the Chinese hamster ovary cell line, CHO-K1 (CCL 61) (*5*), commonly used cellular reagents in our laboratory for the past several years, were also studied. The cells were grown at 37°C in an atmosphere containing 10 percent CO_2 and were transferred by treatment with 0.1 percent trypsin.

Of the 31 cell lines obtained from ATCC, three rat cell lines, two Chinese hamster cell lines, and a pig cell line were found to be producing moderate to high titers of type C virus as determined by supernatant reverse transcriptase assay; two of the fetal cat cell strains from NBRL also showed significant supernatant reverse transcriptase activity in the culture medium (Table 1). The cell cultures were tested for virus production as soon as the initial sample of cells had grown to a confluent monolayer. In addition, apparently virus-free subclones of the NRK line and of the CHO-K1 line spontaneously began to produce type C virus after hundreds of "virus-free" generations in vitro in our laboratory.

Table 1. Properties of mammalian cell lines releasing type C viruses. The reverse transcriptase assay was performed by the method of Ross *et al.* (*17*). The medium (15 ml) from each tissue culture flask was clarified by centrifugation at 12,000*g* for 10 minutes; the virus was then sedimented by centrifugation through 20 percent glycerol (in 0.05*M* tris · HCl, *p*H 7.8; 0.10*M* KCl) at 105,000*g* for 90 minutes at 4°C. The sedimented pellet was suspended in 0.1 ml of buffer [0.05*M* tris · HCl, *p*H 7.8; 0.10*M* NaCl; 10^{-3}*M* dithiothreitol (DTT) containing 0.1 percent Triton X-100]. The virus suspension (0.01 ml) was added to 0.06 ml of reaction mixture containing 0.05*M* tris · HCl, *p*H 7.8; 0.06*M* KCl; 2×10^{-3}*M* DTT; 5×10^{-4}*M* manganese acetate; 0.02 A_{260} units of polyadenylic acid (Miles Laboratories); 0.02 A_{260} units of oligodeoxythymidylate$_{(12-18)}$ (Collaborative Research), and 3×10^{-6}*M* [^{3}H]thymidine triphosphate (40,000 count/min per picomole) (New England Nuclear). Results are expressed as [^{3}H]TMP incorporated (counts per minute) into poly(dT) product during a 60-minute incubation at 37°C (minus that incorporated by the same reaction mixture without template). Supernatants from nonproducer cell lines yield between 500 and 1000 count/min in this assay. For the interspecies gs antigen assay cell monolayers were removed with a rubber policeman, prepared as a 20 percent by volume suspension in phosphate-buffered saline, *p*H 7.2, then subjected to three cycles of freezing and rapid thawing. The resulting preparation was further extracted with ether, which was then evaporated. Radioimmunoprecipitation assays for the mammalian interspecies gs3 antigen were performed as described (*18*). Results are expressed as the highest dilution of a 20 percent suspension giving a clear positive reaction in the assay. Virus assayed for gs antigen was obtained from 20 liters of tissue culture fluid concentrated by isopycnic banding on sucrose gradients.

Species	Cell line	Origin and cell type	Special uses	Assay		
				Supernatant reverse transcriptase activity (10^{-3} count/min)	Interspecies gs antigen (highest positive titer)	
					Cells	Virus
Rat	NRK	Normal kidney; fibroblastic	Transformation assays	914	1 : 8	
	LLC-WRC256 (CCL 38)	Walker carcinoma; epithelial	Tumorigenicity studies	1600	1 : 512	> 1 : 10,000
	R2C (CCL 97)	Leydig cell testicular tumor; epithelial	Control of steroid secretion	298	1 : 8	
	RR1022 (CCL 47)	Schmidt-Ruppin virus induced sarcoma; epithelial	Tumorigenicity studies; rescue of sarcoma virus	189	1 : 64	
Chinese hamster	CHO-K1 (CCL 61)	Ovary; epithelial	Nutritional mutants; regulation of gene expression; cell hybridization	18	1 : 16	
	B14-I50 (CCL 14.1)	Normal peritoneal cells; thymidine kinase negative; fibroblastic	Cell hybridization	22	1 : 2	1 : 512
	NCTC 4206 (CCL 14.2)	Peritoneal cells; fibroblastic	Grows in chemically defined media	5	(−)	
Pig	PK (15) (CCL 33)	Pig kidney; epithelial	Vaccine production	73	1 : 64	> 1 : 10,000
Cat	FFc63RES (NBRL)	Fetus; mixed pool; fibroblastic		15	(−)	
	FFc2K (NBRL)	Fetus; kidney; fibroblastic		183	1 : 32	

Electron micrographs were prepared of the cell lines that produced significant supernatant reverse transcriptase activity. In each case, the electron micrographs demonstrate typical type C virus particles both in the extracellular space and budding from the cell membrane (Fig. 1).

Assays of the virus-producing cell lines (and of several types of virus concentrated from the supernatant) for the type C virus mammalian interspecies group specific antigen (gs3) by a radioimmunoassay are reported in Table 1. Each of the virus-producing cell lines is positive for this mammalian type C virus marker, except when virus titer is low. Radioimmunoassay tests for the species specific antigen of the gs protein (*6*) and inhibition tests of the viral polymerases with antiserums directed against mouse, feline, and woolly monkey type C virus polymerases (*7*) demonstrate that the rat, hamster, and pig viruses are immunologically different from known mouse, feline, and woolly monkey type C viruses.

Repeated attempts were made to transmit the various virus isolates to nonproducer cell lines after ultrafiltration of the tissue culture medium. None of the hamster, rat, or pig viruses were able to replicate on the wide variety of host cell lines tested, including multiple cell lines derived from human, monkey, cat, rat, and mouse cells. (The supernatant reverse transcriptase assay was used to test for viral replication.) The inability of these viruses to infect any of the mammalian cells tested and their distinctly different immunologic properties argue against the possibility that these virus isolates were introduced into the cultures by inadvertent laboratory contamination or by viral contamination of the serums, trypsin, or media used. The viruses from the two cat embryo cell strains, in contrast, readily replicate in primate and human cells. These viruses have antigenic and host range properties closely related to two previously described endogenous cat type C viruses, RD-114 and CCC (*8*).

There have been previous sporadic reports of mammalian cells in vitro spontaneously releasing type C viruses. Certain mouse cell lines produce type C virus with high probability (*2, 3*). Gazzolo *et al.* (*9*) and Bergs *et al.* (*10*) have described type C viruses from rat cell lines, and Armstrong *et al.* (*11*) have described electron microscopic identification of typical type C viruses in pig cell lines, including PK(15) (CCL 33) (*11*). Our data

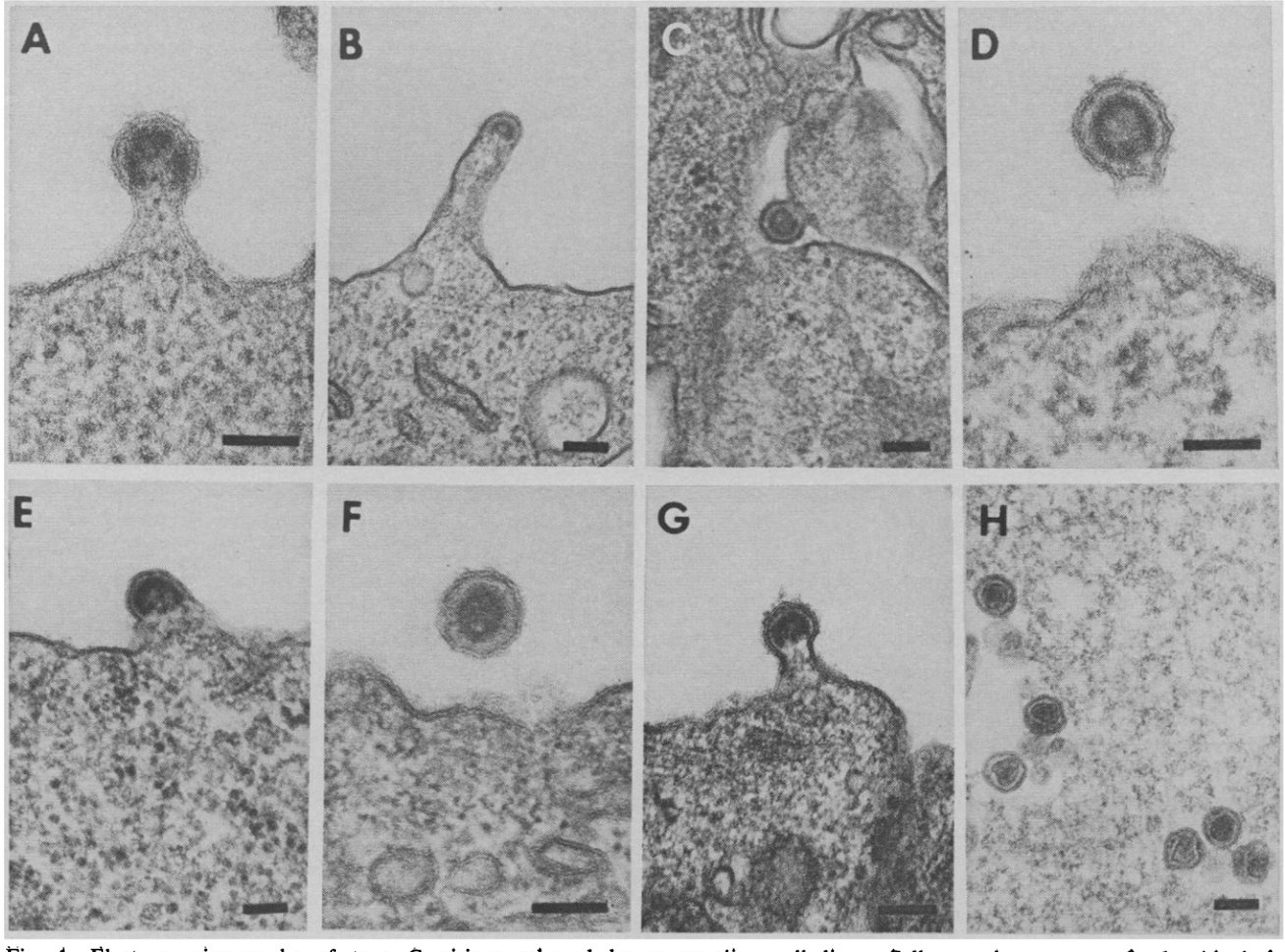

Fig. 1. Electron micrographs of type C virions released by mammalian cell lines. Cell monolayers were fixed with buffered 2 percent glutaraldehyde and then scraped off with a rubber policeman. Cells were then sedimented and fixed in 1 percent buffered osmium tetroxide. After overnight incubation in cold 0.5 percent uranyl acetate, the cells were dehydrated and embedded in Epon. Thin sections were doubly stained in uranyl acetate and lead citrate and examined with a Siemens Elmiskop 1A electron microscope. (A) Budding particle from Chinese hamster NCTC 4206 (CCL 14.2) cell line. (B, C) Budding particles from the Chinese hamster CHO-K1 (CCL 61) cell line 5 days after treatment with IdU (30 μg/ml) for 48 hours. (D, E) Free and budding virions from the rat RR1022 (CCL 47) cell line. (F) A free type C particle from the fetal cat FFc2K cell strain. (G, H) Free and budding particles from the pig PK(15) (CCL 33) cell line. The scale represents 100 nm.

suggest that the spontaneous release of endogenous type C viruses from mammalian cell lines is relatively common. Although most previously described mammalian producer cell lines have been derived from fibroblasts or from lymphoid cells, four of the producer cell lines described here are epithelial in morphology and one is known to secrete steroid hormones. Thus, spontaneous type C virus release is not limited to undifferentiated cell lines or to cells derived from connective or hematopoietic tissue.

The cell lines identified above as spontaneous producers include some of the most commonly used cell lines in cell research and animal virology. Cells that release endogenous type C viruses appear healthy in culture and cannot be distinguished from cells not releasing virus unless the cell cultures are specifically tested for this property. For example, the rat lines NRK, LLC-WRC256, R2C, and RR1022, the pig cell line PK(15), and the cat cell culture FFc2K release type C virus at a rate comparable to the rate of production of laboratory strain murine leukemia viruses from exogenously infected cultures. At present it is not known to what extent high level production of endogenous type C viruses alters the physiology of previously nonproducer cell lines. Nevertheless, it seems plausible that the results and interpretation of many experiments utilizing mammalian cells in vitro could be influenced by this generally unknown factor. Furthermore, since cell lines can spontaneously start producing type C viruses even after hundreds of virus-free cell generations in vitro, such as occurred with the NRK and CHO-Kl cell lines used in this laboratory, any screening process to detect virus-producing cell lines must be applied at frequent intervals.

Various experimental manipulations of virus-free cell lines of chicken, mouse, rat, cat, hamster, and guinea pig origin have been shown to increase greatly the probability of type C virus release (*12*). These include treatment with ionizing radiation, chemical carcinogens, mutagens, and halogenated pyrimidines. The ready induction of hamster type C viruses by 5-iododeoxyuridine (IdU) is a relevant example. One million cells of a virus-free clone of the CHO-Kl (CCL 61) Chinese hamster cell line and of the RPMI 1846 (CCL 49) Syrian hamster cell line were each treated with IdU (30 μg/ml) for 48 hours. Five days after the initiation of treatment, the culture medium of these cell lines was tested for viral reverse transcriptase as described in Table 1. The CHO-Kl cell lines showed 260×10^3 count/min, and the RPMI 1846 cell line, 22×10^3 count/min of [^{3}H]thymidine monophosphate (TMP) incorporated. Untreated control incorporated less than 0.5×10^3 count/min. Electron micrographs of the induced Chinese hamster virus are included in Fig. 1. Labeling of cells with [^{125}I]IdU for use as target cells in cytotoxicity assays is becoming increasingly frequent (*13*). Since the labeling procedures and subsequent incubation closely parallel conditions shown to induce endogenous type C virus in certain cell systems (*12*), and since there is evidence that the release of type C virus can substantially alter the immunogenicity of certain cells (*14*), such experiments seem particularly likely to be influenced by release of type C virus. As another example, long-term treatment with 5-bromodeoxyuridine (BrdU) is commonly used to select mutant cells which lack thymidine kinase. The Chinese hamster peritoneal cell line B-14-I50 (CCL 14.1), which was treated with BrdU for this purpose, is now continuously releasing type C virus; the parent cell line B14FAF28-G3 (CCL 14) is not producing type C virus detectable by the present assay technique. Cells which lack thymidine kinase are commonly used to make interspecies hybrid cell lines with human cells (*15*). The biological properties of type C viruses released from such hybrid cells are not known. However, it seems possible that such virus might be potentially transmissible to humans.

An important consideration, then, is the safety of laboratory workers who commonly handle mammalian cell lines without specific precautions for the handling, storage, and disposal of potentially pathogenic viruses. Although the viruses described, with the exception of the endogenous cat type C virus, have not, in limited testing, been able to replicate in human cells in culture, one cannot be certain that they lack pathogenicity to man. Since type C viruses have been implicated as etiologic agents of leukemias, lymphomas, and sarcomas in a variety of avian and mammalian systems (*1*) and have been identified as etiologic agents of a lower motor neuron disease in mice (*16*), endogenous type C viruses must be regarded as potential biohazards to laboratory personnel. Since mammalian cells in vitro may commonly release such type C viruses in a covert fashion, precautions in handling such cells appear indicated.

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Appendix

Initially tested cell cultures of the following cell lines were not producing detectable levels of type C viruses by the supernatant reverse transcriptase assay used in our investigations. However, since cell lines can begin to produce type C virus after many generations in culture, one cannot be sure these lines will remain virus-free indefinitely.

Name of cell line	ATCC number	Animal source
ATCC cell lines		
BHK-21 (C-13)	CCL 10	Syrian hamster
HaK	CCL 15	Syrian hamster
Don	CCL 16	Chinese hamster
MDBK (NBL-1)	CCL 22	Bovine
MDCK (NBL-2)	CCL 34	Canine
Pt K1 (NBL-3)	CCL 35	Marsupial
Dede	CCL 39	Chinese hamster
Bu (IMR-31)	CCL 40	Buffalo
RPMI 1846	CCL 49	Syrian hamster
E. Derm (NBL-6)	CCL 57	Equine
SIRC	CCL 60	Rabbit
Mv 1 Lu (NBL-7)	CCL 64	Mink
Sf 1 Ep (NBL-11)	CCL 68	Rabbit
Ch 1 Es (NBL-8)	CCL 73	Goat
Pl 1 Ut (NBL-9)	CCL 74	Raccoon
Sp 1 K (NBL-10)	CCL 78	Dolphin
GH_1	CCL 82	Rat
Tb 1 Lu (NBL-12)	CCL 88	Bat
$LLC\text{-}RK_1$	CCL 106	Rabbit
C_6 glial cell	CCL 107	Rat
TRK-1	CCL 143	Rabbit
MH_1C_1	CCL 144	Rat
NBRL fetal cat cell strains		
FFc3Tg		
FFc9WF		
FFc60WF		
FFc2Lu		
FFc2Th		
FFc4K		
FFc72(A) Br		

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Glucodynamic Hormones Modify the Recovery Period after Lateral Hypothalamic Lesions

Abstract. *The period of recovery after bilateral electrolytic lesions of the lateral hypothalamus in rats is shortened if insulin is given for 5 days before surgery, and is lengthened if glucagon is given during the preoperative period.*

Eating behavior ceases completely after bilateral destruction of the lateral hypothalamus (LH) (*1*). If the animals are not properly nursed, they die. However, if the animals are maintained by intragastric feeding, their eating behavior resumes in a well-known recovery pattern (*2*). The length of the recovery period after LH lesions is shortened if the animals are maintained at a reduced body weight before surgery (*3*). This has been taken to indicate that LH lesions reduce a regulatory set point for body weight.

We report here on the hormonal influence on the recovery period that follows LH lesions. We have shown that insulin and glucagon, which are known to modify food intake (*4*), respectively shorten and lengthen the recovery period without necessitating a body weight adjustment before LH lesions are made.

Twenty-one male adult albino rats were adapted for 3 weeks to individual cage housing, Purina rat chow feed, and an illumination cycle of 12 hours of light followed by 12 hours of dark (lights on at 0600). Food intake and body weight were recorded daily throughout the experiment. For 5 days before surgery, seven rats were given 0.2-ml subcutaneous injections of glucagon (0.1 mg at 0000, 0600, 1200, and 1800 hours); seven were injected with 0.2 ml of Semilente insulin (3 units at 0000 and 1200 hours and mock injections at 0600 and 1800 hours); and the remaining seven were injected with 0.2 ml of isotonic saline (at 0000, 0600, 1200, and 1800 hours). In order to maintain body weight during the hormone treatment period, the rats were fed an amount equal to the average amount consumed during the preceding 5 days (*5*). The injections were discontinued 24 hours before surgery.

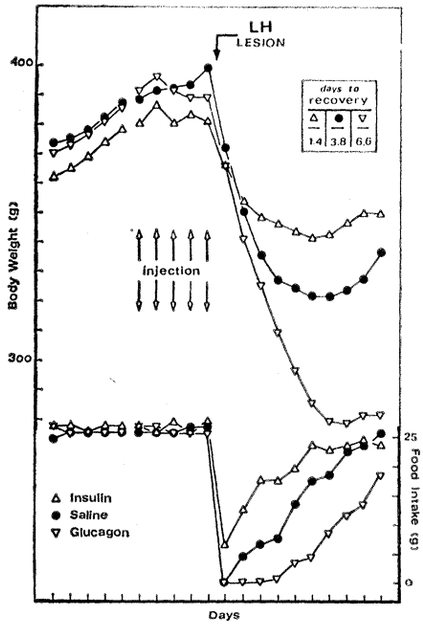

Fig. 1. The influence of injections of insulin, glucagon, and saline on the recovery of feeding after lateral hypothalamic lesions. Recovery of feeding behavior is the time at which animals first begin to eat solid food. Each data point indicates a group mean for 1 day.

Lateral hypothalamic lesions were made under Nembutal anesthesia (50 mg per kilogram of body weight) with the aid of a stereotaxic instrument. Direct anodal current of 1 ma was delivered for 20 seconds through an Insulex-coated stainless steel electrode (0.2 mm in diameter) exposed 0.5 mm at the tip. The stereotaxic coordinates, with the animal's skull in the horizontal position, were 5.6 mm anterior to the interaural line, 2.0 mm lateral to the midsagittal sinus, and 7.7 mm below the dorsal surface of the cortex. The rats were returned to their cages after surgery and their feeding behavior was observed. If an animal had not recovered eating behavior (that is, started to eat solid food) within 7 days, intragastric feeding of milk (5 ml, three times daily) was begun. After recovery of eating behavior, the animals were killed and their brains removed, sliced in sections 60 μm thick, and stained with cresylecht violet for histological verification of the lesion placements.

In every case our lesions encompassed bilaterally the area of the LH and medial forebrain bundle, as well as the most medial edge of the internal capsule at the level of the ventromedial hypothalamus.

Figure 1 summarizes the results. During the hormone treatment, each animal consumed 27 ± 2 g and maintained its preinjection body weight. After LH lesions, the animals that had received saline injections showed the characteristic aphagia and recovered from it in an average of 3.8 ± 2.7 days (*6*). Prior insulin treatment shortened the recovery period to 1.4 ± 1.6 days. Prior glucagon treatment lengthened the recovery period to 6.6 ± 3.1 days. The recovery periods after insulin and glucagon treatments differed significantly in length from that of the saline controls ($P < .05$; two-tailed t-test). An analysis of variance revealed that postsurgical food intake, compared to that of the saline group, was increased by prior insulin treatment and decreased by prior glucagon treatment ($P < .001$; $F = 15.3$; d.f. = 2, 18). The analysis also showed an across-days effect ($P < .01$; $F = 2.5$; d.f. = 9, 162), indicating that the groups reached an eating plateau at different times after the lesion—the insulin group first, the saline