

Susceptibility of rodent cell lines to rat coronaviruses and differential enhancement by trypsin or DEAE-dextran

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Summary. Cell lines of rodent origin were tested for susceptibility to infection with rat coronavirus (RCV), including sialodacryoadenitis virus (SDAV) and Parker's rat coronavirus (PRCV). LBC rat mammary adenocarcinoma cells were susceptible only if the cells were treated with diethylaminoethyl-dextran (DEAE-D). A recent report that RCVs grow well in L2 mouse fibroblast cells was confirmed and expanded. RCV infection of L2 cells was substantially enhanced by treatment of cells with trypsin but not by treatment with DEAE-D. Primary isolation of SDAV from experimentally infected rats was accomplished using trypsin-treated L2 cells. One of 13 additional cell lines tested (rat urinary bladder epithelium, RBL-02) supported growth of RCVs, and growth was slightly enhanced by DEAE-D, but not by trypsin. These refinements of in vitro growth conditions for RCVs should facilitate further studies of their basic biology and improve options for primary isolation.

Introduction

Rat coronaviruses (RCVs), notably sialodacryoadenitis virus (SDAV) and Parker's rat coronavirus (PRCV), infect laboratory rats at high prevalence, cause clinically and economically significant disease and interfere with research using rats [7]. Diagnostic and experimental studies of RCVs have been limited by the lack of a reliable cell line in which to grow the viruses [7]. RCVs have traditionally been grown in primary rat kidney (PRK) cells or infant mouse brain but these techniques require the use of animals and are labor intensive. Although many coronaviruses have fastidious growth requirements, replicating only in organ cultures or cell lines derived from the animal or tissue of origin, cell lines supporting growth of most coronaviruses have been identified [5, 7]. Attempts to grow SDAV in a variety of rodent cell lines, including mouse neuroblastoma (clone N18) cells, C6 rat glial cells, and MHV-susceptible cell lines such as NCTC 1469 cells failed (L. E. Garlinghouse and A. L. Smith,

unpubl. data). Recently Hirano et al. reported that a rat mammary adenocarcinoma cell line (LBC) supported RCV growth [4], and Percy et al. reported that L2 cells supported RCVs [11]. In studies reported here, LBC cells, L2 cells and 13 other cell lines of rodent origin were tested for their susceptibility to RCVs. A rat bladder epithelial cell line (RBL-02) was identified which supports RCV growth. Medium containing trypsin or diethylaminoethyl-dextran (DEAE-D), which have been used to enhance the growth of other coronaviruses [17, 19], also differentially enhanced RCV growth in selected cell lines.

Materials and methods

Cells and media

The cell lines tested and media used are listed in Table 1. HH cells were obtained by *in vitro* transformation of PRK cells [9] by high multiplicity infection with SV-40 [20]. Cell cultures were grown in 25 cm² polystyrene flasks (Corning Glassworks, Corning, NY) at 37°C in a humidified 5% CO₂ atmosphere and were maintained in media containing 100 µg/ml streptomycin and 100 units/ml penicillin. All media and supplements were purchased from GIBCO BRL (Gaithersburg, MD), except MCDB-151 and F-12 media (Sigma Chemical Co., St. Louis, MO). Cells were transferred when confluent using 0.12% trypsin and 0.27 mM EDTA. Two sublines of L2 cells obtained from different laboratories were used (Table 1). Cell lines received from extramural sources except the American Type Culture Collection were tested for *Mycoplasma* spp. and were culture-negative.

Virus

Stocks of SDAV, strain 681 (SDAV-681), and Parker's rat coronavirus (PRCV) were prepared in infant mouse brain [2, 3]. Titration of PRCV and SDAV-681 stocks in primary rat kidney cells [2, 3] yielded titers of 10⁷ TCID₅₀ and 10^{6.5} TCID₅₀ per ml, respectively. The SDAV stock used for concurrent L2 and mouse titration was one infant mouse passage removed from the stock used for cell culture inoculations.

Animals

Cr: ORL Sencar dams with litters were purchased (Animal Genetics and Production Branch, National Institutes of Health, Bethesda, MD) and housed under conditions which met or exceeded standards of the U.S. Public Health Service.

Virus infection

When cell monolayers were approximately 80% confluent, growth medium (Table 1) was removed and cultures were exposed to 1.0 ml of a 1% suspension of virus-infected mouse brain or 1.0 ml of diluent. Fetal calf serum (FCS) was omitted from medium used for trypsin- or DEAE-D-treated cells. Following virus absorption at 37°C for 1 h, 4 ml of medium were added to all flasks. Cultures were observed daily. Cytopathic effect (CPE) was typified by syncytium formation. The percentage of the monolayer that consisted of syncytia was estimated, and all infected and mock-inoculated cultures were harvested at 72 h post-inoculation.

Fluorescence microscopy

Cells no longer adherent to the plastic and cells scraped from the flask surface were washed separately twice, air-dried on Teflon-coated slides (Cel-line Associated, Newfield, NJ) and fixed in ice cold acetone for at least 15 min.

Table 1. Continuous cell lines and media used to grow rat coronaviruses

Name	Species	Tissue	Source	Media ^a
LBC	rat	mammary tumor	Percy [4]	90% MEME, 10% FCS
NRK-49F	rat	kidney fibroblast	ATCC ^b	95% DMEM, 5% FCS
NRK-52E	rat	kidney epithelial	ATCC	45% DMEM, 45% L15, 10% FCS
KNRK	rat	transformed kidney	ATCC	45% DMEM, 45% L15, 10% FCS
4/4 R. M.-4	rat	visceral pleura	ATCC	90% F12, 10% FCS
HH	rat	transformed kidney	Tattersall	95% DMEM, 5% FCS
NBT-II	rat	bladder carcinoma	Leighton [8]	85% MEME, 15% FCS
RBTC-8 (F5)	rat	bladder tumor	Pauli [10]	90% DMEM, 10% FCS
1-RBTC-8 (F6)	rat	bladder tumor	Pauli	90% DMEM, 10% FCS
RBTC-1	rat	bladder tumor	Pauli	90% DMEM, 10% FCS
AY-27	rat	induced bladder tumor	Klaunig [13]	90% MCDB-151, 10% FCS
RBL-02	rat	bladder epithelium	Klaunig	50% F12, 40% DMEM, 10% FCS
3T3 (wt)	mouse	embryo	Tattersall	90% DMEM, 10% FCS
17Cl 1	mouse	3T3 cells	Sturman [18]	90% DMEM, 10% FCS
L2	mouse	fibroblast	Sturman	50% MEME, 40% MEMH, 10% FCS
L2	mouse	fibroblast	Percy [11]	50% MEME, 45% MEMH, 5% FCS

^a MEME Eagle's minimum essential media, FCS fetal calf serum, DMEM Dulbecco's minimum essential media, L15 Liebowitz's L-15 media with L-glutamine, F12 Ham F-12 modified DMEM, MEMH Hank's minimum essential media, MCDB-151 MCDB-151 media

^b American Type Culture Collection, Rockville, MD

Air-dried, fixed cells were incubated with SDAV-immune mouse ascitic fluid or with pooled SDAV-immune serum from experimentally infected rats followed by incubation with fluoresceinated goat anti-species immunoglobulin (Antibodies, Inc., Davis, CA) containing 0.01% Evans's blue [14]. Cells were examined at a magnification of $\times 200$ with a transmitted light fluorescence microscope.

Treatment with trypsin or DEAE-D

Concentrations of porcine parvovirus-free, *Mycoplasma*-free trypsin (GIBCO BRL) from 0.016 $\mu\text{g/ml}$ to 40 $\mu\text{g/ml}$ were added to the absorption and incubation media for L2 (Percy) and RBL-02 cells. Since some tissue homogenates in combination with 16 $\mu\text{g/ml}$ trypsin were toxic to L2 (Percy) cells, 1.6 $\mu\text{g/ml}$ of trypsin was used. Trypsin-treated uninoculated cells were included at each concentration to control for toxicity. Four ml of trypsin-containing medium were added for incubation.

Concentrations of 0, 50, 100, 200 and 400 $\mu\text{g/ml}$ of DEAE-D (Pharmacia Fine Chemicals, Uppsala, Sweden) were added to the pre-absorption and absorption media of LBC, L2 (Percy) and RBL-02 cells to allow sufficient time for action on cell membranes. Duplicate cultures were pre-treated with DEAE-D-containing medium for 4 h, and virus or diluent was added for 1 h at 37°C [19]. DEAE-D containing medium was replaced with growth medium for incubation.

Virus isolation from rat tissues

Nasal washes, lungs and salivary and lacrimal glands from control rats or rats experimentally infected with SDAV-681 were provided by Dr. Eleanor C. Weir, Yale University. The tissues had previously been tested for the presence of infectious virus by intracerebral inoculation of infant mice [21] and had been stored at -70°C until use in this study. One ml of a 3–5% (w/v) tissue homogenate in trypsin-containing medium (1.6 $\mu\text{g/ml}$) was used for inoculation of L2 (Percy) cells. Because lacrimal and salivary gland inocula were toxic to L2 (Percy) cells, they were removed following absorption. Nasal wash and lung inocula were not removed. Cells were examined daily for CPE by an observer uninformed of their status in the prior mouse inoculation study. Cells were subsequently processed and stained for viral antigen.

Concurrent titration of SDAV-681 in infant mice and L2 cells

Serial ten-fold dilutions of stock SDAV-681 virus were inoculated intracerebrally into 2-day old Sencar mice and into flasks containing L2 (Percy) cells in trypsin (1.6 $\mu\text{g/ml}$)-supplemented medium. Mouse mortality at each dilution was recorded for 14 days. L2 (Percy) cells were observed daily for CPE and were stained for viral antigen 72 h after inoculation. Titers expressed as LD₅₀ or TCID₅₀ per ml were calculated [12].

Results

Growth of RCVs in cell lines

L2 (Percy) cells

Antigen-positive cells by immunofluorescence (IF) staining ($< 5\%$) were observed on most attempts in the first passage of SDAV in L2 (Percy) cells. In the second passage, syncytium formation involving about 5% of the monolayer was observed and about 10% of cells were antigen-positive. The proportion of antigen-positive cells increased to about 20% in the third passage. The suscep-

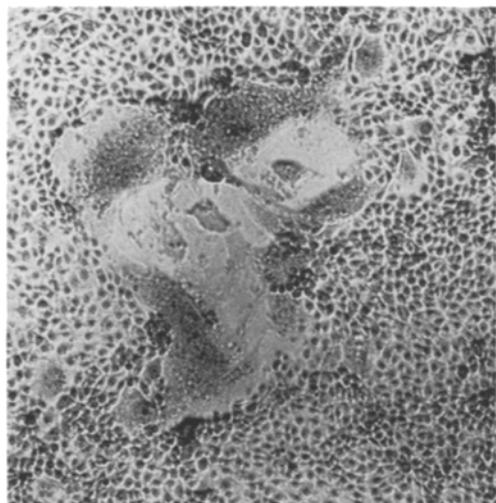


Fig. 1

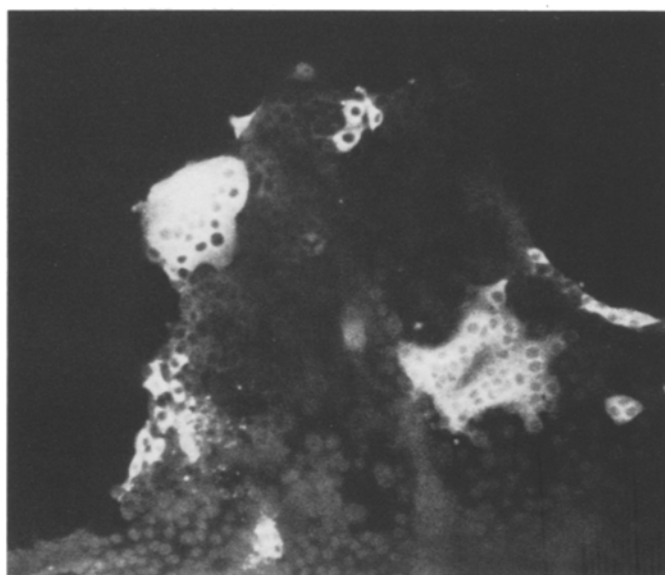


Fig. 2

Fig. 1. Cytopathic effect (syncytium) in unstained RBL-02 cells 48 h post-inoculation with sialodacryoadenitis virus, strain 681 ($\times 350$)

Fig. 2. Multiple fluorescent syncytia formed by sialodacryoadenitis virus, strain 681 in RBL-02 cells at 72 h post-inoculation ($\times 875$)

tibility of L2 (Percy) cells to PRCV was erratic. Less than 5% of the monolayer formed syncytia, and antigen-positive cells ($< 5\%$) were seen on the first passage in half of the attempts, whereas PRCV growth was not detected in the remaining attempts. Seven additional passages did not enhance PRCV growth.

RBL-02 cells

SDAV-681 and PRCV grew in the first passage in RBL-02 cells. Syncytia involving 5% of the monolayer formed within 48 h (Fig. 1) and antigen-positive cells (about 5%) were seen by IF staining (Fig. 2). CPE was not seen in the second passage, and fewer cells contained antigen. Virus growth was not detected in the third through seventh passages.

LBC cells

Repeated attempts, including 11 blind passages, failed to reveal growth of SDAV-681 or PRCV in LBC cells. CPE was not seen, and virus antigen was not detected.

Other cell lines

RCV growth was not detected in other cell lines listed in Table 1. At least 2 passage attempts of both SDAV-681 and PRCV were completed in all cell lines.

Table 2. Effect of trypsin on growth of rat coronaviruses in L2 and RBL-02 cells

Cell line	Treatment	Passage no.	Virus strain			
			SDAV		PRCV	
			CPE ^a	antigen ^b	CPE	antigen
L2 (Percy)	none	1	–	+ ^c	+	+ ^d
		2	+	+	–	–
		3	+	+	–	–
	trypsin ^e	1	+	+	+	+
		2	–	–	–	–
RBL-02	none	1	+	+	+	+
		2	–	+	–	–
		3	–	–	–	–
	trypsin ^f	1	–	–	–	+
		2	–	–	–	–

^a Syncytia formation

^b As detected by indirect immunofluorescence of non-adherent or adherent cells

^c One fifth of attempts unsuccessful

^d One half of attempts unsuccessful

^e 1.6 µg/ml

^f 0.16 µg/ml, higher concentrations of trypsin were toxic to RBL-02 cells

Enhancement of RCV growth by trypsin

Trypsin enhanced growth of RCVs in L2 (Percy) cells in the first passage (Table 2), but not in LBC (data not shown) or RBL-02 cells. After treatment with trypsin, about 10% of inoculated L2 (Percy) cells developed medium-sized syncytia, whereas syncytia were not seen in untreated SDAV-681-inoculated L2 (Percy) cells. Trypsin treatment also increased the proportion of antigen-positive L2 (Percy) cells to about 10%. Increased trypsin concentration up to 16 µg/ml increased the proportion of infected L2 (Percy) cells, but concentrations higher than 16 µg/ml were toxic. RBL-02 cells were treated with a 10-fold lower concentration of trypsin than L2 (Percy) cells due to a lower toxicity threshold.

Enhancement of RCV growth by DEAE-dextran

Concentrations of 50–400 µg/ml of DEAE-D substantially enhanced growth of RCVs in LBC cells, enhanced growth marginally in RBL-02 cells, and did not affect growth in L2 (Percy) cells (Table 3). Viral antigen was not detected in mock-treated, inoculated LBC cells, but about 10% of non-adherent cells were virus antigen-positive in treated, inoculated cultures following exposure to

Table 3. Effect of DEAE-dextran on growth of rat coronaviruses in L2, RBL-02 and LBC cells^a

Cell	Virus	DEAE-dextran ($\mu\text{g/ml}$)				
		0	50	100	200	400
L2 (Percy)	SDAV	±	±	±	—	—
	PRCV	—	±	—	—	—
RBL-02	SDAV	±	±	±	+	±
	PRCV	—	±	+	+	+
LBC	SDAV	—	+	+	+	+
	PRCV	—	±	+	+	+

^a As detected by viral antigen in adherent or non-adherent cells, — not detected, ± less than 5% cells positive, + >5% cells positive

200–400 $\mu\text{g/ml}$ of DEAE-D. Enhancement of RCV growth in DEAE-D-treated RBL-02 cells was marginal and evident only by examination of non-adherent cells stained for virus antigen.

Use of trypsin-treated L2 (Percy) cells for primary isolation and quantification of SDAV-681

In a single attempt to isolate SDAV-681 from the lung of an experimentally infected rat, syncytia and viral antigen were first seen after 6 passages in untreated L2 (Percy) cells. In contrast, syncytia and antigen were observed in the first passage in trypsin-treated L2 (Percy) cells. Based on this finding, a small study was initiated to ascertain the sensitivity of trypsin-treated L2 (Percy) cells for primary isolation of RCVs.

Table 4. Comparison of trypsin-treated L2 (Percy) cells and mouse inoculation for isolation of SDAV from experimentally infected rats

Tissue	L2 cells			Mouse inoculation
	CPE ^a	IF ^b	combined	
Lung	10/15 ^c	10/15	12/15	15/15
Lacrimal gland	2/7	2/7	2/7	6/7
Salivary gland	2/7	5/7	5/7	6/7
Nasal wash	6/9	7/9	7/9	8/9

^a Syncytia formation

^b Indirect immunofluorescence

^c Number positive/number examined

Virus isolations were attempted using tissue homogenates previously tested for SDAV-681 by infant mouse inoculation (Table 4). CPE and virus antigen were observed in 57% and 69%, respectively, of cultures exposed to virus-positive tissues. The highest concordance between mouse and L2 (Percy) cell inoculation (88%) was obtained using nasal wash as the inoculum.

A single stock of SDAV was quantified concurrently in infant mice and trypsin-treated L2 (Percy) cells. The titer of stock SDAV-681 was $10^{6.4}$ LD₅₀ per ml in infant mice and $10^{4.5}$ TCID₅₀ per ml in trypsin-treated L2 (Percy) cells.

Discussion

Limited RCV growth was demonstrated in untreated L2 (Percy) and RBL-02 cells, but not in other cell lines tested, including LBC cells and an L2 subline obtained from another source. A recent observation of SDAV antigen in transitional epithelium of experimentally infected athymic rats [21] led us to assess rat bladder epithelial cell lines for growth of RCVs, and RBL-02 cells supported RCV growth on initial passage. Of the cell lines and treatments tested, L2 (Percy) cells treated with trypsin supported maximal growth of RCVs as evidenced by syncytium formation and viral antigen. RCV growth in L2 (Percy) cells was not enhanced by trypsin treatment of later passages when the enzyme was added at the time of virus inoculation. Adding trypsin at a different time in the RCV replicative cycle might enhance RCV growth in successive passages. As previously reported by Percy et al. [11], repeated passage of SDAV-681 in untreated L2 (Percy) cells increased viral growth. Repeated passage of other animal-adapted RCV strains in L2 (Percy) cells may accomplish adaptation and increased virus yield.

Trypsin, but not DEAE-D, enhanced RCV growth in L2 (Percy) cells. The mechanism by which trypsin increases RCV growth in these cells is not known. However, trypsin enhances mouse hepatitis virus (MHV) plaque formation [15, 19] and cleaves the 180k E2 glycoprotein of MHV A59 into two 90k fragments [17]. Cleavage is associated with a two-fold increase in infectivity and with activation of the cell-fusing activities of virions *in vivo* [16, 17]. Therefore, trypsin could enhance RCV infectivity by cleaving its E2 protein analogue.

Trypsin treatment of L2 (Percy) cells permitted primary isolation of SDAV from tissues of infected rats without repeated passage. The use of trypsin-treated L2 (Percy) cells to screen tissues for RCVs would significantly reduce animal use and expense. However, L2 (Percy) cells were about $2 \log_{10}$ less sensitive than infant mice based on comparative titration of SDAV-681. This may have been due to adaptation of stock SDAV-681 to infant mouse brain by virtue of multiple prior passages in mice. If trypsin-treated L2 (Percy) cells are inherently less sensitive than infant mice, tissues that are virus-negative by assay in these cells should be re-tested in mice to eliminate false negatives. Uninfected L2 (Percy) cells are morphologically heterogeneous and sensitive to tissue toxicity; therefore, antigen detection which is less subjective than interpretation of morphologic changes should be the definitive test used after cell culture inoculation.

Using trypsin-treated L2 (Percy) cells, virus in lacrimal gland was more difficult to detect than virus in other tissues tested. A possible explanation for low isolation rates from lacrimal gland is the presence of a trypsin-inhibitor that is not present in other tissues. However, litters of mice inoculated with lacrimal gland homogenates tended to have lower mortality rates and deaths that occurred later than in groups inoculated with other tissues (data not shown). This suggests that lacrimal gland samples may simply have contained lower concentrations of virus.

RCV growth was increased in LBC and RBL-02 cells treated with DEAE-dextran, a chemical that enhances virus binding and uptake by altering electrostatic interactions [1]. This result suggests that virus attachment and/or penetration are the limiting steps for RCV growth in these cell lines. DEAE-D also enhances infection of cells with MHV-3 [19], although the mechanism is unknown. Studies of binding and internalization with MHV-JHM have shown, however, that several rat cell lines share a common deficiency at the level of virus internalization [7]. DEAE-D may act by enhancing RCV binding to LBC and RBL-02 cells, thereby facilitating viral entry into cells that otherwise support RCV growth.

Little is known about the replication and biochemistry of RCVs largely due to their fastidious *in vitro* growth requirements. Our described refinements of *in vitro* growth conditions for RCVs should facilitate both *in vitro* and *in vivo* studies. Repeated passage of rat-adapted RCV isolates and RCV growth in L2 (Percy) and RBL-02 cells may increase virus yield and permit more sophisticated studies of antigenic relatedness among RCV isolates. Use of trypsin-treated L2 (Percy) cells should facilitate *in vivo* studies of RCV pathogenesis requiring RCV isolation.

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