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Optimization of in vitro growth conditions for enterotropic murine coronavirus strains

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Abstract

Enterotropic mouse hepatitis virus (MHV) strains have been difficult to grow in cell culture. In an attempt to develop an efficient in vitro cultivation system for enterotropic MHV strains (MHV-RI and MHV-Y), 8 murine cell lines were inoculated with MHV-RI- or MHV-Y-infected infant mouse intestinal homogenates and screened for the production of cytopathic effects. MHV-RI and MHV-Y consistently produced cytopathic effects only in J774A.1 cells. Both strains produced titers of $> 10^6$ $TCID_{50}$/ml in subsequent passages in J774.1 cells. MHV strains -1, -3, -A59, -JHM, -S and -DVIM also produced high-titer viral stocks in J774A.1 cells. Therefore J774A.1 cells are the first cells found that support the replication of these 8 enterotropic and respiratory MHV strains. After passage in J774A.1 cells, MHV-RI and MHV-Y could infect previously non-susceptible cell lines (17Cl-1, CMT-93, N18 and NCTC 1469), though cytopathic effects were often negligible with MHV-RI. MHV-Y, but not MHV-RI, grew in L2(Percy) cells. Using L2(Percy) cells, an agarose overlay and Giemsa staining, MHV-Y could be quantified by plaque assay. Infant mouse bioassays, plaque assays and cell culture infections were compared for their sensitivity in detecting MHV-Y in infected intestinal homogenates and cell supernatants.

Keywords: Murine coronavirus; Mouse hepatitis virus strain RI and Y; Enterotropism; J774A.1 cell; Plaque assay

Mouse hepatitis virus (MHV), a singular name for several murine coronaviruses, causes a wide spectrum of clinical syndromes ranging from subclinical infection to enteritis, hepatitis, encephalitis and death. MHV strains can be segregated into two biotypes, based on their initial site of replication: respiratory (polytropic) and en-

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terotropic (Barthold, 1986). Following oronasal inoculation, respiratory MHV strains initiate replication in the upper respiratory tract and then disseminate to multiple organs if the mouse is adequately susceptible due to age, genotype or immune status (Barthold, 1986; Compton et al., 1993). In contrast, replication of enterotropic MHV strains, such as MHV-RI and MHV-Y, is largely restricted to the intestinal mucosa, with minimal or no dissemination to other organs (Barthold, 1987; Barthold et al., 1993).

Respiratory MHV strains, because they are easily propagated in vitro, have been studied intensively serving as the prototypes for coronavirus molecular biology and as models of viral hepatitis and encephalitis (Compton et al., 1993). On the other hand, enterotropic MHV strains, such as MHV-RI and MHV-Y, are the most common strains found in contemporary mouse colonies (Barthold, 1986), but little work has been done on their pathogenesis or molecular biology since they have been difficult to grow in vitro. MHV-RI was originally isolated in CMT-93 cells from a nude mouse intestine (Barthold et al., 1985). MHV-Y was originally isolated in NCTC 1469 cells from the intestine of a naturally infected infant mouse with acute typhlocolitis (Barthold et al., 1982). High-titered stocks of MHV-RI and MHV-Y have traditionally been generated in infant mouse intestines. Although stocks of MHV-RI can be generated in CMT-93 cells and stocks of MHV-Y can be generated in NCTC 1469 cells, viral titers produced are low (10^3–10^5 $TCID_{50}$/ml) (Barthold et al., 1982, 1985). More importantly, virus was recoverable from only a small proportion of MHV-infected intestinal homogenates using NCTC 1469 or CMT-93 cells. Therefore, virus production in tissues of mice infected with enterotropic MHV strains traditionally has been measured using an infant mouse bioassay, a costly and time-consuming procedure (Barthold, 1987). Recently, detection of viral genome in intestinal homogenates using reverse transcriptase-PCR or monoclonal antibody solution hybridization have been used for detection, but not quantitation, of enterotropic MHV production in infected mouse tissues (Homberger et al., 1991; Kunita et al., 1992; Casebolt and Stephensen, 1992; Yamada et al., 1993). We report the development of efficient in vitro propagation and quantitation methods for enterotropic MHV strains.

Eight murine cell lines (17Cl-1, CMT-93, J774A.1, L2.p176, L2(Percy), NCTC 1469, N18 and WEHI-279), which support the replication of MHV-A59, were inoculated with intestinal stocks of MHV-RI or MHV-Y in an attempt to find a cell line which would efficiently propagate enterotropic MHV strains. 17Cl-1 cells, obtained from L. Sturman (Albany, NY), were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS). J774A.1, obtained from American Type Culture Collection (Rockville, MD), were grown in RPMI medium 1640 supplemented with 10% FBS. NCTC 1469 cells, obtained from A. Smith (Yale), were grown in NCTC medium supplement with 10% FBS. WEHI-279 cells, obtained from A. Smith (Yale), were grown in 95% Clicks/EHAA medium supplemented with 50 μm 2-mercaptoethanol and 5% FBS. CMT-93 cells, obtained from American Type Culture Collection (Rockville, MD); N18 cells, obtained from A. Smith (Yale); L2(Percy) cells, obtained from D. Percy (Guelph, Canada) and L2.p176 cells (Gaertner et al., unpublished data) were grown in Dulbecco's minimal essential medium/Leibowitz-15 medium supplement with 10% FBS. Cells were incubated at 37°C and observed daily for cytopathic effects (CPE) for up to 5 days postinoculation (p.i.).

Table 1
Growth of intestinal stocks of MHV-Y and RI in cell culture

Cells	Hours p.i.	MHV-Y		MHV-RI	
		CPE [a]	Titer [b]	CPE	Titer
J774A.1	72	4	4.75	3	3.75
L2(Percy)	72	3	5.36	0	$\leqslant 1.5$
L2.p176	72	3	3.75	0	$\leqslant 1.5$
N18	48	3	3.25	0	2.50
	72	ND	ND	0	3.75
NCTC 1469	48	1	4.75	0	$\leqslant 1.5$
	72	1	4.50	0	$\leqslant 1.5$
17Cl-1	72	0	4.50	0	$\geqslant 5.5$
CMT-93	72	0	$\leqslant 1.5$	0	$\leqslant 1.5$
WEHI-279	72	0	$\leqslant 1.5$	0	$\leqslant 1.5$

Each well of a 24-well plate was inoculated with 250 μl of 1% MHV-RI- or MHV-Y-infected infant mouse intestinal homogenates. Cell layers were washed at 3 h p.i. and 1 ml of fresh medium was added.

[a] CPE: 0, no CPE observed; 1, < 10% cells involved; 2, 10–50% cells involved; 3, 50–90% cells involved; 4, > 90% cells involved; ND, not determined.

[b] Titers are expressed as $\log_{10}$ $TCID_{50}$/ml of cell supernatant measured in J774A.1 cells. At 4–5 h p.i. all cell supernatants possessed titers of $\leqslant 1.5$

In repeated attempts, only J774A.1 cells consistently sustained substantial CPE (greater than half the cells involved in syncytial formation) following inoculation with MHV-RI and also with MHV-Y (Table 1). When inoculated with either MHV-RI or MHV-Y, greater than 75% of J774A.1 cells formed syncytia within 72 h p.i. MHV-Y, but not MHV-RI, also produced substantial CPE in L2(Percy), L2.p176 and N18 cells. Somewhat surprisingly, MHV-Y did not cause substantial CPE in NCTC 1469 cells and MHV-RI did not cause substantial CPE in CMT-93 cells, the cell lines in which they were isolated originally. Virus production in cells inoculated with intestinal stocks of MHV-RI or MHV-Y was quantified in J774A.1 cells. Triplicate wells of a 96-well plate of confluent J774A.1 cells were inoculated with serial 10-fold dilutions of viral supernatants and cells were assessed for CPE, primarily syncytia formation, at 48 and 72 h p.i. $TCID_{50}$s were calculated using the method of Reed and Muench (1938). Both MHV-RI and MHV-Y produced detectable titers in J774A.1 cells (Table 1). Although, substantial CPE was not seen in MHV-Y-infected 17Cl-1 or NCTC 1469 cells or in MHV-RI-infected 17Cl-1 or N18 cells, detectable viral titers were produced in these cell lines (Table 1). Clearly for MHV-RI, and to a lesser degree for MHV-Y, the ability of an MHV strain to cause syncytia did not correlate with the ability to produce infectious virus. Only J774A.1 cells were capable of producing both substantial CPE and viral titers with both MHV-RI and MHV-Y, making them a very useful cell line for the recovery of enterotropic MHV strains from intestinal homogenates.

Stocks of MHV-RI and MHV-Y generated by multiple passages through J774A.1 cells had titers of 10^6–10^9 $TCID_{50}$/ml. J774A.1-adapted stocks of MHV-RI and MHV-Y acquired the ability to grow in NCTC 1469 cells, the cell line in which MHV-Y was originally isolated in, resulting in syncytia formation involving greater than 90% of the cells and producing titers of 10^7–10^9 $TCID_{50}$/ml. J774A.1-adapted stocks of MHV-RI and MHV-Y routinely produced viral titers of less than 10^4 $TCID_{50}$/ml in

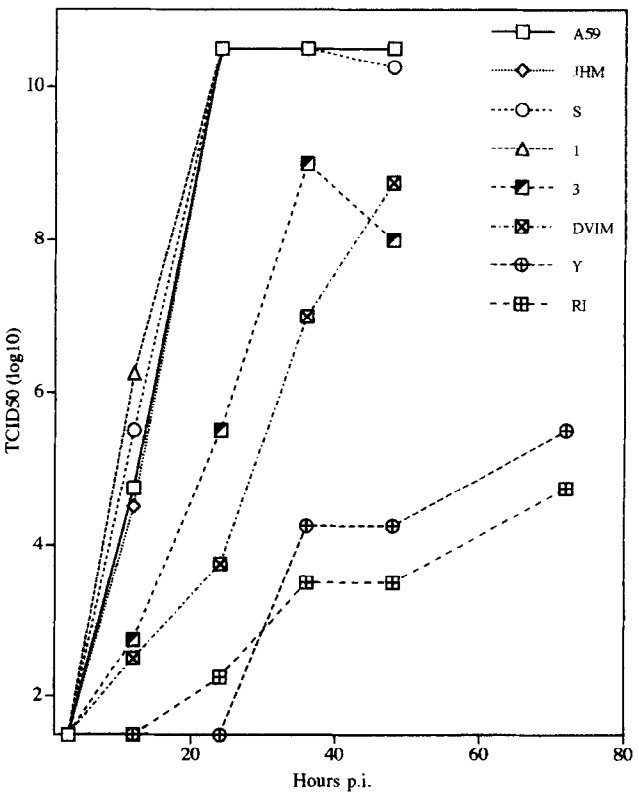

Fig. 1. Growth of MHV strains in J774A.1 cells. Each well of a 24-well plate was inoculated with 20 $TCID_{50}$ of a J774A.1-adapted MHV stock (previously passed from 1 to 3 times in J774A.1 cells). Cell layers were washed at 3 h p.i. and 1 ml of fresh medium was added. Cell supernatants were removed at 12, 24, 36, 48 or 72 h p.i. Titers are expressed as $\log_{10}$ $TCID_{50}$/ml of cell supernatant measured in J774A.1 cells.

CMT-93 cells, the cell line in which MHV-RI was originally isolated in, with only minimal CPE. J774A.1-adapted stocks of MHV-Y gained the ability to fuse 17Cl-1 cells resulting in syncytia formation involving greater than 90% of the cells and producing titers of greater than 10^8 $TCID_{50}$/ml. J774A.1-adapted stocks of MHV-RI produced negligible CPE in 17Cl-1 and N18 cells, but produced viral titers of greater than 10^5 $TCID_{50}$/ml in both cell lines.

We also investigated whether other MHV strains were able to grow in J774A.1 cells. MHV-1, -3, -A59, -JHM and -S were obtained from American Type Culture Collection (Rockville, MD) and MHV-DVIM was obtained from K. Holmes (Bethesda, MD). J774A.1 adapted stocks of MHV-3, -JHM, -A59, -DVIM and -1 were generated by a single passage in J774A.1 cells of mouse brain stocks of MHV-3 and MHV-JHM, NCTC 1469 stocks of MHV-A59, MHV-S and MHV-DVIM and a 17Cl-1 stock of MHV-1. The 5 respiratory MHV strains (MHV-A59, -JHM, -S, -1, and -3) produced titers of $\geqslant 10^9$ $TCID_{50}$/ml within 36 h p.i. (Fig. 1). In contrast, MHV-RI and Y had produced titers of only $10^{4.75}$ and $10^{5.5}$ $TCID_{50}$/ml at 48 h p.i. (Fig. 1). MHV-DVIM, a

strain of MHV which possesses characteristics of both respiratory and enterotropic MHV strains (the ability to cause both severe enteritis and hepatitis in infant Sencar mice) (Barthold and Compton, unpublished data) was intermediate in its virulence in J774A.1 cells (Fig. 1). The ability of all 8 MHV strains to grow in J774A.1 cells makes J774A.1 cells ideal for generating recombinant enterotropic/respiratory MHV strains in cell culture. J774A.1 cells will also be useful for comparing viral replication rates of enterotropic and respiratory MHV strains in a single permissive cell line.

The ability of MHV-Y to produce syncytia in L2(Percy) and L2.p176 cells allowed us to modify published plaque assay methods for MHV-JHM and SDAV, which utilize L2 cells, to develop a plaque assay for MHV-Y (Lucas et al., 1977; Gaertner et al., 1993). Briefly, L2(Percy) cells were grown in 12-well plates until confluency. Each well was rinsed once with phosphate-buffered saline (PBS) and inoculated with 500 μl of diluted virus. After 1 h adsorption, the virus inocula was removed and 2 ml of 0.95% agarose in basal minimal medium was added. Plates were incubated at 33, 37 or 39°C in an 5% CO_2 atmosphere. Cell layers were fixed at 2 or 3 days p.i. by the addition of 2 ml of 10% buffered formalin and incubated at room temperature for at least 16 h. Formalin and agarose were removed, the cell sheet was rinsed once with PBS, stained with Giemsa for 10–15 min, and rinsed with distilled water. Plaques were counted and titers were expressed in $\log_{10}$ plaque-forming units (PFU)/ml of infected cell supernatant or 10% intestinal homogenate. MHV-Y produced equal numbers of plaques in L2(Percy) and L2.p176 cells, but MHV-A59 produced much smaller and substantially fewer (> 100-fold less) plaques in L2.p176 cells than in L2(Percy) cells. Therefore, we chose to use L2(Percy) cells as our standard cell line for plaque assays. Addition of trypsin to the overlay media (at 2.5 μg/ml) increased MHV-Y plaque size slightly, but had little or no effect on plaque numbers and resulted in weakly staining monolayers. Pretreatment of cells with DEAE-D (75 μg/ml) and inclusion of DEAE-D (75 μg/ml) in viral inoculation media increased MHV-Y plaque numbers approximately two-fold, but resulted in fragile monolayers which often peeled off of the plates. Use of newly confluent cells rather than subconfluent cells or cells which had been confluent for more than 2 days resulted in the cell layers which stained the most evenly and plaques which were the easiest to visualize. Incubation of MHV-Y or MHV-A59 plaque assays at 37 or

Table 2
Comparison of viral quantitation methods

MHV-Y passage history	$TCID_{50}$	PFU	ED_{50}
Intestine-multiple	3.5	5.9	5.2
Intestine-multiple, J774-2	5.8	5.0	< 2.5
Intestine-multiple, J774-3, intestine-1	6.2	5.9	4.2
Intestine-multiple, NCTC-7, J774-4	6.3	5.9	2.8
Intestine-multiple, NCTC-7, J774-4, intestine-1	5.5	4.7	2.6

$TCID_{50}$ and PFU measurements are the average of 2 or 3 experiments. $TCID_{50}$ is expressed as $\log_{10}$ tissue culture infectious $dose_{50}$ in J774A.1 cells/ml of cell culture supernatant or intestinal homogenate and PFU is expressed as $\log_{10}$ plaque forming units in L2(Percy) cells/ml of cell culture supernatant or intestinal homogenate. ED_{50} measurements are the result of a single experiment and are expressed as $\log_{10}$ enteritis $dose_{50}$ in neonatal Sencar mice/ml of cell culture supernatant or intestinal homogenate.

39°C for 2 days led to equal numbers of plaques, whereas incubation of plaque assays at 33°C for 3 days resulted in a decreased number of plaques (~ 10-fold) for both MHV-Y and MHV-A59. Investigation into the possible cold sensitivity of MHV-A59 and MHV-Y revealed that even though virus titers in cell supernatants from infections at 33°C were equal or greater than those produced at 37°C, fewer cells were involved in syncytia formation. Therefore the decreased number of plaques observed at 33°C was probably due to the formation of plaques too small to be detected. A plaque assay was not developed for MHV-RI as J774A.1 and NCTC 1469 cells, the two cell lines in which MHV-RI causes substantial CPE, were easily detached during plate washings, resulting in patchy monolayers.

A comparison of the sensitivity of different virus quantitation methods (ED_{50}, $TCID_{50}$, PFU) was undertaken (Table 2). Enteritis doses were determined using groups of four 24- to 48-h-old Sencar mice inoculated orally with 10 μl of serial 10-fold dilutions of viral stock. Mice were killed at 48 h p.i., ilea, ceca and ascending colons were removed, processed for histology and examined microscopically for MHV lesions (syncytia, necrosis and inflammation) (Barthold et al., 1993). Infant mouse ED_{50}s were calculated using the method of Reed and Muench (1938). The titer of an MHV-Y stock passed exclusively in mouse intestines was equivalent as measured by plaque assay and infant mouse bioassay, but was several magnitudes higher than the viral titer as measured in cell culture infection of J774A.1 cells (Table 2). In contrast titers of viral stocks serially passed in cell culture were equivalent as measured by plaque assay and tissue culture infection, but were several orders of magnitude higher than that measured in the infant mouse bioassay. This result suggests that passage of intestinal stocks of virus in cell culture leads to the selection of a subgroup of viruses which replicate well in cell culture, but poorly in the mouse. A single passage of cell culture-adapted MHV-Y in mouse intestine did not substantially alter the relative sensitivity of the different assay methods, suggesting that a single passage in the mouse is not sufficient to amplify a subpopulation of viruses from the cell culture adapted viral stocks which can grow efficiently in the mouse. The relative insensitivity of the $TCID_{50}$ assay compared to the plaque assay in detecting MHV-Y in intestinal homogenates may be a result of differing fusion abilities of viruses in intestinal stocks in L2(Percy) and J774A.1 cells. Approximately 75% of the L2(Percy) cells inoculated with 1% infant intestinal homogenates of MHV-Y were involved in syncytial formation at 48 h p.i., whereas only 20–25% of the J774A.1 cells inoculated with 1% infant intestinal homogenates of MHV-Y were involved in syncytial formation at 48 h p.i. J774A.1-adapted stocks of MHV-Y, had produced greater than 90% syncytial formation in both L2(Percy) and J774A.1 cells at 48 h p.i. This result indicates that growth of MHV-Y stocks in J774A.1 cells may have selected for a subpopulation of viruses which are highly fusagenic for both cell lines.

The identification of J774A.1 cells as a cell line capable of sustaining visible CPE and producing detectable viral titers with both MHV-RI and MHV-Y, indicates that this cell line should be included in attempts to recover enterotropic MHV strains from intestinal homogenates. The development of efficient methods of in vitro propagation and quantification of enterotropic MHV strains should aid in the molecular characterization of these viruses. The ability of J774A.1 cells to grow all 8 MHV strains tested

makes them an ideal cell line for generating recombinant enterotropic/respiratory MHV strains and for comparing replication rates of enterotropic and respiratory MHV strains in a single permissive cell line.

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