

In vitro Growth Characteristics and Heterogeneity of Mouse Hepatitis Virus Type 3

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With 2 Figures

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Summary

The *in vitro* virus yield of MHV3 reached 10^7 PFU/ml in mouse DBT cells infected with a virus suspension in HEPES-buffered medium containing DEAE-dextran. The virus titer was 10^6 PFU/ml in the presence of $10 \mu\text{g}$ actinomycin D/ml. MHV3 grown in DBT cells gave three peaks of density (1.10 — 1.14 g/cm^3 , 1.18 — 1.20 g/cm^3 , and 1.25 — 1.31 g/cm^3) in sucrose gradients. All these peaks retained infectivity.

Introduction

The similarities in the host-parasite relationship between hepatitis produced in mice by the murine coronavirus, mouse hepatitis virus type 3 (MHV3) (13), and human serum hepatitis have encouraged several laboratories to study both the pathological and immunological MHV3-infection of mice (5, 7, 8). Some of the biochemical properties of MHV3 have been determined (10, 11), although little is known about the molecular events during replication (7). This is undoubtedly due to difficulty in growing MHV3 in cell cultures, although the growth of MHV3 in some cells has been described (6, 7, 10). We previously reported a plaque assay in a mouse cell line (12) and in this paper we describe an improved cultivation method of MHV3 in the same cell line. Furthermore, we show heterogeneity of MHV3 particles by sucrose gradient analysis.

Materials and Methods

Cell Culture, Virus Strain, and Infectivity Assay

The cell-line SR-CDF1-DBT (DBT), originating from a mouse brain tumor (2) was grown in Eagle's minimum essential medium (MEM) supplemented with 10 to 15 per cent calf serum (CS) and 10 per cent tryptose phosphate broth. MEM was buffered with sodium bicarbonate or N-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid

(HEPES). The American Type Collection Strain of MHV 3 was used after three cycles of plaque purification in our laboratory. Plaque assays were performed on DBT-monolayers in 3.5-cm diameter petri dishes as described previously (12).

Growth Experiment at Various Temperatures

DBT-monolayers, grown in 30-ml plastic bottles, were infected with MHV 3 at a multiplicity of infection (MOI) of 0.1 PFU per cell in the presence of 25 $\mu\text{g/ml}$ diethylaminoethyl-dextran (DEAE-D). The addition of 25–100 $\mu\text{g DEAE-D/ml}$ to the virus inoculum increased the virus yield twofold (data not shown). After a 45-minute adsorption at 37° C, the monolayers were covered with 4 ml of HEPES-buffered MEM (pH 7.2) containing 2 per cent CS, and incubated at various temperatures. Incubation was performed in a water-bath controlled to $\pm 0.1^\circ\text{C}$. After the selected time duplicate DBT-bottles were collected and the culture fluids were pooled, centrifuged and the supernatant was assayed for infectivity (cell-free virus titer; CFV titer). The remaining monolayers were covered with 4 ml of phosphate buffered saline, frozen and thawed twice, and assayed for infectivity (cell-associated virus titer; CAV titer).

Radioactive Labelling and Centrifugation of Virus

To label virus RNA, DBT cells, grown in 2000-ml roller bottles, were infected with MHV 3 at MOI of 0.02 PFU per cell. After a 45-minute adsorption at 37° C the cells were given HEPES-buffered MEM (pH 7.2) containing 5 μg actinomycin D/ml and incubated at 37° C for 6 hours. The culture fluids were then removed and cells were covered with MEM containing 2 per cent dialysed CS, 5 μg actinomycin D/ml, and 5 $\mu\text{Ci/ml}$ of ^3H -uridine (sp. act. = 24 Ci/mm), and incubated for 12 hours at 37° C. To label virus protein, DBT cells were infected as described above and were incubated at 37° C for 17–18 hours in HEPES-buffered MEM containing either methionine reduced to 10 per cent of the usual concentration and ^{35}S -methionine (2 $\mu\text{Ci/ml}$, sp. act. = 575 mCi/mm), or a mixture of ^3H -leucine (2 $\mu\text{Ci/ml}$, sp. act. = 30 Ci/mm), ^3H -valine (2 $\mu\text{Ci/ml}$, sp. act. = 26 Ci/mm), ^3H -phenylalanine (2 $\mu\text{Ci/ml}$, sp. act. = 30 Ci/mm), and ^3H -lysine (1 $\mu\text{Ci/ml}$, sp. act. = 22 Ci/mm).

The virus was pelleted by centrifugation at $23,000 \times g$ for 40 minutes in a Beckman 42.1 rotor and was resuspended in TNE buffer (0.01 M Tris-hydrochloride, 0.1 M NaCl, and 0.001 M EDTA, pH 6.8). This was layered on a 15–65 per cent linear sucrose gradient (w/w) and centrifuged at $40,800 \times g$ for 90 minutes in a Beckman SW 27 rotor (velocity centrifugation). Some fractions were pooled, dialysed, layered on a 15–65 per cent sucrose gradient and centrifuged at $40,800 \times g$ for 16–18 hours in an SW 27 rotor. In Exp. 3 (see Table 2) culture fluids were layered on to a cushion consisting of 10 ml of 15 per cent sucrose made in TNE buffer and centrifuged at $40,800 \times g$ for 60 minutes in an SW 27 rotor to remove fine cellular debris prior to velocity centrifugation. The fractions were collected from the bottom of the tube and assayed for acid-precipitable radioactivity and infectivity. The densities were determined by weighing 100-microliter samples.

Results

Virus Yields and One-Step Growth Curves at Various Temperatures

It has been reported that the yields of avian and porcine coronaviruses were seriously influenced by the pH of the medium and that the virus yield was increased by using a medium buffered with HEPES (1, 9). Our preliminary experiments have shown that a HEPES-buffered medium (pH 7.2) produced about a 10-fold increase in virus yield in DBT cells compared with a medium buffered with sodium bicarbonate (pH 7.2). The CAV and CFV of MHV 3 grown in DBT cells were higher using MEM containing 20 mM HEPES at pH 7.2 than at pH 6.6 (data not shown). For this reason HEPES-buffered medium (pH 7.2) was used.

The viral yield was compared at various temperatures between 31°—42° C (Table 1), in order to determine the optimal temperature of MHV3-growth in DBT cells. The highest titer in culture fluids was obtained when the infected cells were incubated at 35° C. One-step growth experiments were performed at 35°, 37°, 40°, and 42° C. No virus was obtained at 42° C. Both CFV and CAV were highest at 35° C at 12 and 18 hours after incubation, although the difference between the yield at 35° and at 37° C was not large. The CFV titer was slightly higher than the CAV titer at 35° and 37° C after an 18-hour incubation (Fig. 1).

Table 1. *Effect of temperature on the virus yield*

Hours after incubation	Incubation temperature (° C)							
	31°	33°	35°	37°	39°	40°	41°	42°
18 hours	10 ^{4.15} ^a	10 ^{6.45}	10 ^{6.94}	10 ^{6.41}	10 ^{5.57}	10 ^{4.85}	10 ^{2.00}	n.d. ^b

^a Cell-free virus titer (PFU/ml)

^b Not detected

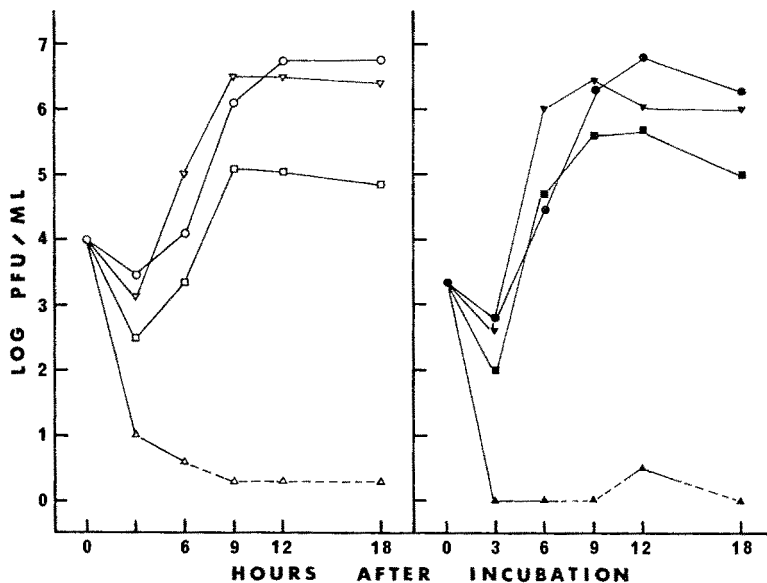


Fig. 1. One-step growth curves of MHV 3 at various temperatures

At selected times duplicate DBT-monolayers infected with MHV 3 at MOI of 0.1 PFU per cell were collected and the culture fluids pooled, centrifuged, and assayed for infectivity (cell-free virus titer, left panel). The original volume of phosphate-buffered saline was added to the monolayers, which were then frozen and thawed twice and assayed for infectivity (cell-associated virus titer, right panel)

Symbols: Virus growth at 35° C (○ & ●), 37° C (▽ & ▼), 40° C (□ & ■), and 42° C (△ & ▲)

Effects of Actinomycin D on Cellular RNA Synthesis and Virus Production

The time course and the dose response of actinomycin D on RNA-synthesis in DBT cells were studied. RNA-synthesis in DBT cells was decreased by over

95 per cent at one hour and by over 98 per cent at two hours after incubation, when 2.5 or 5 $\mu\text{g/ml}$ of actinomycin D was added to the medium (Fig. 2, left). The yield of MHV3 in DBT cells was reduced by up to 10-fold in the presence of actinomycin D (1–10 $\mu\text{g/ml}$) (Fig. 2, right). However, the inhibitory effect of the drug seemed to be secondary to an adverse effect on DBT cells.

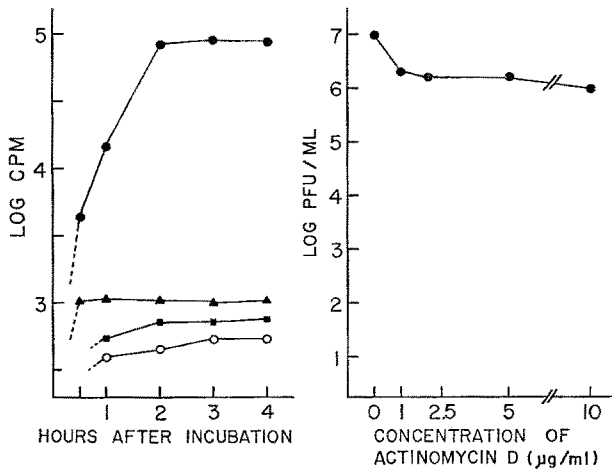


Fig. 2. Effect of actinomycin D on the cellular RNA-synthesis and virus yield

Left: DBT cells in wells of a microplate (Nunc; Roskilde, Denmark) were incubated at 37° C in MEM containing both ^3H -uridine (4 $\mu\text{Ci/ml}$) and actinomycin D, or MEM containing ^3H -uridine alone. At the indicated times cells were disrupted by adding sodium dodecyl sulfate (final concentration = 1 per cent) and the acid-precipitable radioactivity was determined

Right: DBT cells were infected with MHV3 at MOI of 0.1 PFU per cell and covered with MEM containing different concentrations of actinomycin D. After incubation for 18 hours at 37° C, the cell-free virus titer was determined

Symbols; no actinomycin D (●); actinomycin D at 0.5 $\mu\text{g/ml}$ (▲), 2.5 $\mu\text{g/ml}$ (■), and 5 $\mu\text{g/ml}$ (○)

Sucrose Gradient Analysis of MHV3

One peak of radioactivity (CPM-peak) at 1.25 g/cm^3 and two peaks of infectivity (PFU-peak) at 1.25 and 1.20 g/cm^3 were obtained, when concentrated MHV3 was centrifuged in a 15–65 per cent sucrose gradient at 40,800 $\times g$ for 16 hours. The discrepancy between the densities of the PFU- and CPM-peaks suggested that the MHV3 samples were composed of heterogeneous virus particles. Velocity centrifugation was then done prior to equilibrium centrifugation. The peak fractions from the bottom and top of velocity centrifugation tubes were taken and centrifuged at 40,000 $\times g$ for 16–18 hours in a 15–65 per cent sucrose gradient. Results are shown in Table 2. The bottom fractions produced single CPM-peaks corresponding to PFU-peaks of density 1.26–1.131 g/cm^3 . The top fractions produced 2–4 CPM-peaks and 3–4 PFU-peaks at the bottom (1.25–1.31 g/cm^3), middle (1.18–1.20 g/cm^3), and top (1.10–1.14 g/cm^3) of the gradients. These CPM- and PFU-peaks coincided with each other.

Table 2. *Equilibrium centrifugation of MHV 3 in 15—65 per cent sucrose gradients*

Experi- mental number	Radioisotope used	Fraction	Peak of radioactivity		Peak of infectivity	
			Num- ber of peaks	Density (g/cm ³)	Num- ber of peaks	Density (g/cm ³)
1	Mixture of ³ H-amino acids	Bottom	1	1.26	1	1.26
		Top (PFU) ^a	2	1.19	3	1.20
				1.14		1.18
					1.14	
2	³ H-uridine	Bottom	1	1.30	1	1.30
		Top (PFU)	0		2	1.29
						1.20
		Top (CPM) ^b	4	1.30	3	1.30
				1.20		1.20
	1.18			1.10		
			1.14			
3	³⁵ S-methionine	Bottom	1	1.31	1	1.31
		Top (PFU)	3	1.30	4	1.29
				1.19		1.27
				1.11		1.19
					1.11	

Total infectivities in starting materials: Exp. 1: 10^{7.38} PFU, Exp. 3: 10^{7.89} PFU

^a Peak of infectivity

^b Peak of radioactivity

Discussion

MHV3 did not replicate in DBT cells as efficiently as MHV2 (2), but the method we propose is an improvement on previous methods. By giving DEAE-D to DBT cells with the virus suspension, MHV3 reached a higher titer than in untreated cells. DBT cells in HEPES-buffered MEM (pH 7.2) produced more virus than those in MEM buffered with sodium bicarbonate. Furthermore the virus yield reached 10⁶ PFU/ml even in the presence of 5—10 µg/ml of actinomycin D which within two hours inhibited cellular RNA synthesis by over 98 per cent. The optimal temperature of virus production was found to be 35° C, although MHV3 seemed to grow well within the range of 33°—37° C. Furthermore, DBT cells were easy to handle compared with mouse macrophages and mouse embryo cells. Therefore, DBT cells seem to offer a convenient cell system in which the biochemical properties of MHV3 can be investigated and so we attempted to purify MHV3 from them.

We have not succeeded in purifying MHV3, although our sucrose gradient experiments indicated that MHV3 particles were heterogeneous. Two main groups of virus particles were separated by velocity centrifugation. The bottom peak produced one PFU-peak corresponding to a CPM-peak, but the top peak formed two to four further peaks. Thus, MHV3 grown in DBT cells seemed to contain particles of different densities; high density (HD; 1.25—1.31 g/cm³), intermediate density (ID; 1.18—1.20 g/cm³), and low density (LD; 1.10—1.14 g/cm³). The LD and ID particles appear to be made from membrane-bound MHV3

and complete MHV3 particles, respectively, because a substantial amount of infectivity was detected in them and the densities of LD and ID particles were comparable to those of membrane-bound human coronavirus (HCV)/OC43 (3) and of intact coronavirus (3, 4, 7). The HD group was composed of unusual virus particles which were infectious but more dense than is usual with coronaviruses. It has been reported that HCV/229E particles are easily deprived of their petal-like projections (4), and that treatment with NP40 increases the density of the virus particles from 1.19 to 1.27 g/cm³ (4). We therefore postulate that the HD group might be composed of incomplete virions which had been damaged during centrifugation. However the reason why the HD group retained infectivity remains unknown.

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