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Growth of Murine Cytomegalovirus in Various Cell Lines

KWANG SOO KIM AND RICHARD I. CARP

Department of Microbiology, New York State Institute for Research in Mental Retardation, Staten Island, New York 10314

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Murine cytomegalovirus (MCMV) was capable of infecting and replicating in both primary and continuous cell lines obtained from various species. In African green monkey kidney (BSC-1) cells, primary rabbit kidney cells, and baby hamster kidney (BHK-21) cells, there were cytopathic effects (CPE) and virus replication upon initial exposure of cells to virus. In primary fetal sheep brain (FSB) cells, L cells, and rabbit kidney (RK-13) cells, it was necessary to subculture the infected cells one or more times before appearance of CPE and replication of virus. In the case of the infected FSB cultures, it was found that the virus effect could be induced if subculturing were accomplished by trypsinization but did not occur if cells were subcultured by scraping. FSB-grown virus replicated better in FSB than in mouse embryo fibroblast (MEF) cells. The CPE produced in all of the above cell lines was similar to that observed in MEF infected with MCMV. The virus grown in different cell lines was completely neutralized when mixed with several reference sera prepared in rabbits or mice. The populations of virions released from infected MEF and FSB cells were compared by isopycnic centrifugation in potassium tartrate, and no differences were revealed in the buoyant densities of the populations. Human embryonic brain cells, human embryonic kidney cells, a human lung fibroblast cell strain (WI-38), HeLa, and Hep-2 were not susceptible to MCMV.

The cytomegaloviruses are medium-sized deoxyribonucleic acid viruses with a lipid-containing envelope that are morphologically and chemically similar to the herpes group of viruses. The groups differ in two characteristics. (i) The cytomegaloviruses replicate more slowly and produce lower yields of infectious particles, and (ii) the cytomegaloviruses are reported to have a marked specificity with regard to susceptible hosts in contrast to the broad host range of the herpesviruses (4). Exceptions to the host specificity of cytomegaloviruses have been reported in a few instances, such as the limited growth of murine cytomegalovirus (MCMV) in primary rabbit kidney (PRK) cells (G. D. Hsuing, *personal communication*), the growth of vervet cytomegalovirus in human fibroblast cells (5), and the adaptation of a field mouse cytomegalic strain to growth in a number of cell types (6). In the last work noted, the origin of the virus replicating in the cells of the heterologous species was not ascertained. In the course of experimentation initiated with the dual purpose of discovering latent systems for cytomegalovirus and of finding a host system that would produce high virus yields, it was observed that a murine strain of cytomegalovirus was capable of infecting and replicating in a wide range of cell systems. In this communication the interaction of MCMV with a number of cell types is documented, and evidence is presented indicating that the virus produced in the cells from heterologous species was similar to the virus produced in mouse cells.

MATERIALS AND METHODS

Cells. Primary mouse embryo fibroblast (MEF) cells were prepared from 15- to 18-day mouse embryos of the CFW strain by trypsinization. The cells were seeded either in flasks (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.), 35-mm petri dishes (Falcon Plastic) or 32-oz Brockway bottles. The growth medium, unless otherwise stated, was Eagles' basal medium containing fetal calf serum at a concentration of 10%. All media contained penicillin and streptomycin at a concentration of 100 units/ml and 100 μg/ml, respectively.

Primary fetal sheep brain (FSB) cells were prepared from minced FSB tissues and planted in growth medium. A number of the explants attached to the flasks, and eventually cells from the explants formed monolayers. The monolayers were subcultured with

trypsin, and the cells were used for making flask and plate cultures.

PRK cells were prepared by the procedures described by Schmidt (7).

Human embryonic kidney (HEK), human embryonic brain (HEB), and African green monkey kidney (BSC-1) cells were obtained from Flow Laboratories, Inc., Rockville, Md. These cells and L cells, HeLa S-3, rabbit kidney (RK-13), and Hep-2 cells were propagated in the standard growth medium; whereas, baby hamster kidney (BHK-21; clone-13) cells were grown in standard growth medium containing, in addition, Tryptose-phosphate broth at a 10% concentration.

Virus. MCMV was obtained from Donald Henson of the Section of Infectious Diseases, National Institute of Neurological Disease and Stroke. In addition, the Smith strain (lot no. 3) obtained from the American Type Culture Collection was used. In those instances in which this strain was used, it is specifically referred to as the Smith strain. The virus strain obtained from Henson and maintained in this laboratory was identical to the Smith strain in all characteristics and was neutralized by sera prepared against the Smith strain. For the growth of MEF-passaged virus, MEF cultures in 32-oz Brockway bottles were infected, and the fluid and cells were harvested when 75% or more of the cells exhibited cytopathic effect (CPE). At the time of harvest, the infected cultures were either treated with three cycles of freezing and thawing or with ultrasonic vibrations for 1 min (model W140D Bronson Sonifier) at 20 kc operating at a setting of step 5. The virus strains were used following 5 to 10 passages in MEF cells.

Plaque titration. Plaque assays were accomplished by inoculating virus onto cell monolayers contained in small dishes (35 by 10 mm; Falcon Plastic) or flasks (25 cm^2; Falcon Plastic) and allowing the virus to absorb for 1 to 1.5 hr at room temperature, before overlaying with Eagle's basal medium containing 1% Noble Agar (Difco) and 5% fetal calf serum. A second and third overlay of media were applied on day 3 and day 6, respectively. The composition of these overlays was the same as the first, except that neutral red at a concentration of 1:10,000 was included in the third overlay. Plaque counts were done on day 7 without the aid of any magnifying lens. The plaque sizes varied from 1.5 to 2.5 mm in diameter, and the average diameter was about 2.2 mm.

Virus neutralization. Serial twofold dilutions of antisera were prepared. Equal volumes of the serum dilution and the stock virus, calculated to contain approximately 100 plaque-forming units (PFU), were mixed. The virus-antibody mixtures were incubated at 37 C for 1 hr and immediately titrated on monolayers to determine PFU.

Preparation of antisera. Rabbits were immunized with virus that had been concentrated 20 times. The virus was pelleted by centrifuging at 25,000 rev/min for 2 hr in a model B-60 ultracentrifuge (type A147 rotor; International Equipment Co., Needham Heights, Mass.). The pellet was washed once with phosphate-buffered saline (PBS) and resuspended to $\frac{1}{20}$ of the original volume in PBS and inoculated intramuscularly at 3- to 5-day intervals for a series of 10 injections. Ten days after the last injection, the rabbits were bled. Mice (50) were given two sublethal doses of MCMV 1 month apart and were bled 10 days after the second injection.

Preparation of tritiated thymidine-labeled virus. Inocula containing approximately 1 PFU per cell were allowed to absorb for 90 min at room temperature. The infected cells were then incubated at 37 C in growth medium containing 0.3 μCi of tritiated thymidine per ml. (^{3}H-thymidine activity was 11.9 Ci/mmoles.)

Concentration and partial purification of ^{3}H-thymidine virus. Harvested virus (50 ml) was exposed to ultrasonic vibrations for 1 min. The lysate was pelletized in a model B-60 centrifuge, type A147 rotor, at 25,000 rev/min for 2 hr. The pellet was resuspended in complete growth media and again treated with ultrasonic vibrations for 1 min and pelletized. The second pellet was resuspended in one-fifth of the original volume of complete media and used as concentrated virus after an additional treatment with ultrasonic vibrations for 1 min.

For isopycnic banding of the virus, 20 to 50% potassium tartrate in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride (*p*H 7.2) was used. The final *p*H of the 20 and the 50% tartrate in Tris-chloride was 7.5 and 8.3, respectively. Continuous gradients were formed with a gradient former (Beckman Instruments, Inc., Fullerton, Calif.) in tubes (0.5 by 2 inch). The gradient was overlaid with 0.1 ml of labeled virus and centrifuged at 60,000 rev/min for 18 hr at 4 C in a centrifuge, (type SW-65K rotor; Beckman Instruments, Inc.). Fractions (0.3 ml) were collected from the bottom of the tube by using the piercing unit made by Buchler Instruments. PFU, H3 disintegrations, and refractive index were determined for each fraction. H3 disintegrations were measured in a Picker Liquimat-330. The density of each fraction was determined by referring the refractive index reading to the standard refractive index charts previously determined by using potassium tartrate in Tris-chloride buffer.

RESULTS

Infectivity of MCMV after repeated cycles of freezing and thawing and treatment with ultrasonic vibration. The effect of ultrasonic vibration on virus titer was compared with the effect of repeated freezing and thawing. The results (Tables 1 and 2) indicated that repeated cycles of freezing and thawing had virtually no effect upon virus titer. The above experiments were repeated with the Smith strain, and similar results were obtained. In contrast, in comparison to untreated samples, infectivity increased after 30 sec of sonic treatment and remained consistently higher throughout the 8-min treatment period. Because of the above data and because of the greater convenience of ultrasonic treatment, this was the method of choice employed for releasing cell-associated virus and for dispersing virus particle aggregates.

TABLE 1. *Infectivity of MCMV after repeated cycles of freezing and thawing*

Freeze-thaw cycles[a]	Log 10 PFU[b]/ml	Difference from starting sample (log 10 PFU/ml)
0	6.09	0
1	6.04	−0.05
2	6.10	+0.01
3	5.87	−0.22
4	6.06	−0.03
5	6.15	+0.06
6	6.02	−0.07
7	6.07	−0.02
8	6.06	−0.03
9	5.80	−0.29
10	6.19	+0.10

[a] Freezing done at −20 C in a chilled ethanol bath; thawing done in tap water = 1 cycle.
[b] Plaque-forming units.

TABLE 2. *Infectivity of MCMV after treatment with ultrasonic vibration*

Time of treatment at 20-kc sonic waves (min)	Log 10 PFU[a]/ml	Difference from starting sample (log 10 PFU/ml)
0	6.18	0
0.5	6.45	+0.27
1	6.41	+0.23
2	6.43	+0.25
4	6.45	+0.27
8	6.31	+0.13

[a] Plaque-forming units.

Replication and CPE of MCMV in cells from mouse, hamster, rabbit, and monkey. The replication of MCMV in BSC-1, BHK-21, PRK, and MEF cells was examined in a series of virus growth experiments. Confluent monolayers of each cell type were prepared in plastic flasks. Medium was removed from the flasks, and the virus inoculum [0.5 ml per flask at a multiplicity of infection (MOI) of 0.5] was allowed to absorb at room temperature for 1 hr. Then fresh medium was placed into the flasks, and the cultures were incubated at 37 C. At the indicated time intervals, one flask from each cell line was removed and maintained at −70 C until all the cultures had been harvested. After thawing and sonic treatment for 1 min, the samples were tested for PFU. The virus grew in each of the heterologous cell lines, although maximum yields were lower than the yield of MCMV in MEF cells (Table 3). The differences between the virus inoculum and the total yield of virus per culture were 60-, 2-, and 8-fold for the BSC-1, BHK-21, and PRK cells, respectively. In the case of BHK-21 cells, the difference between the total virus obtained in the culture and the virus present in the cultures during the eclipse phase of the virus cycle (24 hr postinfection) was 20-fold. The cytopathic changes in the heterologous cells were similar to the CPE seen in MEF cells and were characterized by rounding, swelling, and aggregation of cells. Some cells disintegrated, leaving holes in the monolayer. The cultures were stained with May-Gründwald-Giemsa, and almost all cells contained distinctive eosinophilic intranuclear inclusions.

Infection of FSB cells with MCMV. When primary FSB cells were exposed to MCMV at an input MOI of 0.1, there was no detectable CPE in the following 2 weeks. After 2 weeks the cells were subcultured with trypsin. One week after subculturing the FSB cells showed typical CPE, similar to that seen in infected MEF cells. The CPE included swelling, rounding, and aggregation of cells. Typical intranuclear inclusions were seen in the affected cells. When FSB cells were exposed to virus under identical conditions, but were subcultured without enzyme treatment, the cells did not show any sign of CPE. These cells were observed for 5 weeks before being discarded as negative. The FSB-adapted virus grew upon serial passages in FSB cells. The titer of passage 18 was 1.15×10^7 PFU per ml, which is comparable to the highest virus titer obtained from MEF cells infected with unpassaged MCMV. The virus also grew well if passed back into MEF cells where it produced typical CPE. FSB-grown virus was titrated simultaneously in MEF and FSB cell monolayers to compare plaquing efficiency, and it was found that the plaque counts were much higher (90%) in FSB than in MEF monolayers (Table 4).

Infection of L cells and RK-13 cells with MCMV. L cells and RK-13 cells grown in flasks (Falcon Plastic) were exposed to virus at an MOI of 0.01. The flasks were observed for CPE and checked for virus production by PFU determination in MEF cells. When the infected monolayers became very heavy, the cells were subcultured into three new flasks, and again the cells were observed daily for CPE. The experiment included a total of seven subculturings, each at 5- to 10-day intervals. Samples from each of the passages were stored at −20 C prior to virus quantitation. By the fourth passage, CPE began to appear in L cells. By the sixth and seventh passages, the L cells were not multiplying at a sufficient rate to make monolayers in 10 days, and most of the cells showed CPE.

The virus yield from each of the L cell passages was determined in MEF cells (Table 5). FSB-adapted virus initiated the yield of virus and the

TABLE 3. *Replication and cytopathic effect (CPE) of MCMV in cell lines and primary cell cultures derived from various species*

Tissue culture designation[b]	CPE and virus production[a]								
	Primary culture	No. of subculture[c]							Highest yield obtained (PFU[d]/ml)
		1st	2nd	3rd	4th	5th	6th	7th	
BSC-1	+								6.0×10^6
BHK-21	+								2.0×10^5
PRK	+								8.25×10^5
MEF	+								7.0×10^7
FSB	−	+							1.15×10^7
L	−	−	−	+	+	+	+	+	8.0×10^4
RK-13	−	−	−	+	+	+	+	+	4.0×10^4
Hela	−	−	−	−	−	−	−	−	0
WI-38	−	−	−	−	−	ND	ND	ND	0
HEB	−	−	−	−	−	ND	ND	ND	0
HEK	−	−	−	−	−	ND	ND	ND	0

[a] Symbols: +, CPE and virus replication; −, no CPE and no virus replication; ND, not done.

[b] Abbreviations: BSC-1, African green monkey kidney; BHK-21, baby hamster kidney; PRK, primary rabbit kidney; MEF, mouse embryo fibroblast; FSB, fetal sheep brain; RK-13, rabbit kidney; WI-38, human lung fibroblast cell strain; HEB, human embryonic brain; HEK, human embryonic kidney cells.

[c] Each subculture was done at approximately 3 to 5 days for L, Hela, and RK-13 cells and at about 10-day intervals for HEB, HEK, and WI-38 cells.

[d] Plaque-forming units.

TABLE 4. *Plaquing efficiency of fetal sheep brain (FSB)-adapted virus in FSB and mouse embryo fibroblast (MEF) monolayers*

Virus passage no. in FSB	PFU[a]/ml for virus titrated in:	
	MEF monolayers	FSB monolayers
FSB-2	1.75×10^4	1.45×10^5
FSB-11	2.10×10^4	2.16×10^5

[a] Plaque-forming units.

TABLE 5. *Replication of MCMV in L cell cultures infected with virus grown in fetal sheep brain (FSB) and mouse embryo fibroblast (MEF) cells*[a]

Date subcultured	No. of subcultures	PFU/ml obtained from L cell cultures infected with MCMV grown in:	
		FSB (passage-9)	MEF
15 November	0	0	0
20 November	1	0	0
24 November	2	5	0
28 November	3	6	0
5 December	4	880	0
10 December	5	4,500	125
18 December	6	55,000	65
24 December	7	81,000	1,500

[a] Subculturing was done by dispensing the cells from one flask into three of the same size flasks. Plaque-forming units (PFU) were determined in MEF monolayers.

development of CPE by the third passage, i.e., earlier than virus effect was noted with MEF-passaged MCMV. In addition, the yield at each passage was higher with FSB-passaged virus. In general, plaque titers increased with increasing numbers of subcultures of the infected cells. The results obtained with infected RK-13 cells were very similar to those shown for L cells. The development of CPE in the passages of these cells and in all others tested is summarized in Table 3.

Infection of HEB, HEK, HeLa, Hep-2, and WI-38 cells with MCMV. HEB, HEK, HeLa, Hep-2, and human lung fibroblast cell strain (WI-38) cells were grown and infected as described for other cell types. The cells were examined for CPE daily and subsequently checked for virus by plaque titration. After infection the cells were maintained in flasks and subcultured four times in 6 weeks. During this time no CPE or virus yield was detected (Table 3).

Characteristics of MCMV grown in various cell lines. Neutralization tests with MCMV grown in various cell lines and anti-MCMV sera prepared in rabbits and mice were done as described in Materials and Methods. As shown (Table 6), viruses grown in various cell lines were completely neutralized by anti-MCMV sera at high

TABLE 6. *Neutralization of MCMV grown in cells from various species*

Serum	Source of virus[c]	Cont.	Plaque counts per plate[a] serum dilution							
			1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280
A[b]	MEF	100	0	0	0	0	0	1	1	11
A	FSB	320	0	0	0	0	0	0	4	9
A	AGMK	47	0	0	0	0	0	2	ND	ND
A	PRK	69	0	0	0	2	1	5	ND	ND
A	RK-13	6	0	0	0	0	0	2	ND	ND
A	BHK-21	20	0	0	0	0	0	0	2	13
Pre-A	MEF	14	13	ND	ND	ND	ND	ND	ND	ND
Pre-A	FSB	13	12	ND	ND	ND	ND	ND	ND	ND
B	MEF	82	0	0	0	0	5	20	ND	ND
B	FSB	171	0	0	0	0	18	77	82	ND
Pre-B	MEF	67	68	ND	ND	ND	ND	ND	ND	ND
Pre-B	FSB	86	76	ND	ND	ND	ND	ND	ND	ND
C	MEF	160	0	0	3	18	34	86	116	125
C	FSB	170	0	0	0	20	100	89	188	200
Pre-C	MEF	16	13	ND	ND	ND	ND	ND	ND	ND
Pre-C	FSB	39	55	ND	ND	ND	ND	ND	ND	ND

[a] Used duplicate plates. ND, not done.

[b] Symbols: A, anti-MCMV prepared in rabbits with mouse embryo fibroblast (MEF)-grown virus. Serum was absorbed with normal MEF cells before use; B, anti-MCMV prepared in rabbits with fetal sheep brain (FSB)-grown virus. Serum absorbed three times with FSB cells before use; Pre-A, preimmunized rabbit serum A; Pre-B = preimmunized rabbit serum B; C, immunized mouse serum. A group of mice were inoculated ip with a sublethal dose of MEF-grown virus as described in Materials and Methods; Pre-C, pool of normal mouse serum.

[c] Abbreviations: PRK, primary rabbit kidney; RK-13, rabbit kidney; BHK-21, baby hamster kidney cells.

dilutions. For example, the virus obtained from FSB cells was neutralized to the same extent as MEF-grown virus by serum produced in rabbits against either FSB- or MEF-grown virus and by mouse serum produced against MEF-grown virus. The results, therefore, indicated that all the viruses were identical with regard to their susceptability to neutralization by serum prepared against MEF-grown murine virus, and that there was no evidence for a nonneutralizable fraction of virus following growth in other host cells.

Populations of ^{3}H-thymidine-labeled virions were released from infected MEF and FSB cells by ultrasonic treatment for 2 min and were then compared with regard to their buoyant density as described in Materials and Methods. There was no difference in the density spectra of the labeled virions obtained from FSB and from MEF cells. The peak of labeled virions occurred at a density of 1.22 to 1.23 for virions obtained from each cell type.

DISCUSSION

Mouse cytomegalic virus has been found to cross the species barrier in that it was capable of replicating in monkey, sheep, rabbit, and hamster cells. The initiation of replication in PRK, BHK-21, and BSC-1 cells occurred upon initial exposure of cells to virus, whereas with FSB, RK-13, and L cells a number of subcultures were required before there was evidence of virus replication. Strict host restriction for MCMV was evidenced only with human cell lines and strains where the mouse virus failed to replicate. The reason(s) that most cytomegalic viruses are restricted to tissue culture cells of the specific host species is not understood. Likewise, the reason(s) for the differences in host-virus interaction obtained with MCMV in the present study is not clear. Differences in cell receptors are frequently described as an explanation for variations in cell susceptibilities (2, 3, 8). The importance of cell receptors in the case of MCMV is suggested by the results with FSB-passaged material in which virus replication occurred after subculturing of infected cells by enzyme treatment, but not when cells were subcultured by scraping. An alternate hypothesis is that virus particles gain entry into cells but fail to cause CPE or go through a complete replication cycle. The use of virus adsorption studies, various enzyme treatments, and the cell fusion technique should help to elucidate the role of receptor sites in cell resistance to MCMV.

The properties of MCMV described in this report are more suggestive of the herpesviruses than of standard cytomegaloviruses. Thus, the

comparatively high yield of virus and the lack of strict host specificity with regard to replication are markedly different from the host-virus interactions noted for most cytomegaloviruses (4).

The cytomegalic virus produced in different cell lines was identical to the original mouse strain produced in mouse cells with regard to the following characteristics. (i) The cytopathic changes produced by the strains were identical. (ii) The viruses were all neutralized by the same reference sera. (iii) The buoyant densities of the labeled virions obtained from infected MEF and FSB cells were identical. The results strongly suggest that the virus produced in the various cells were the progeny of the mouse virus and not a latent virus of the particular cells being infected. In addition, in the case of the BSC-1, PRK, and BHK-21 cells, the cells became infected upon initial exposure to MCMV, and it is, therefore, highly unlikely that the rapid development of CPE and virus replication could have been related to a latent virus infection of these cells.

A series of experiments was attempted in which cells from various nonhuman species were infected with human cytomegalic virus. These experiments included subculturing infected cells with trypsin, as was done with the FSB cells infected with MCMV. In no instance was there any CPE or detectable virus replication.

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