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Propagation of MM Virus in L Cells

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MM virus (mouse-brain stock) replicated to a limited extent in L cells without cytopathic effects; the average yield was less than 1 plaque-forming unit/cell. Passage in BHK-21 cells resulted in MM virus [MM(BHK)] which replicated to high titers (200 to 300 plaque-forming units/cell) in L cells with complete cytopathic effects. Appearance of mature MM(BHK) virus in L-cell cultures begins 4 hr after infection and is completed by 8 hr. Release of mature virus was slow (less than 1% at 8 hr) but was completed by 24 hr.

MM virus was first isolated and described by Jungeblut and Dalldorf (5). It is classified with the encephalomyocarditis (EMC) group of viruses which includes Columbia SK, EMC, F, Mengo, and SVW. Members of this group appear to be immunologically indistinguishable strains of a single virus (8), but different strains have different growth requirements (6). Although MM virus has been carried almost exclusively by mouse brain passage, in vitro growth of the virus has been demonstrated in various tissues of mice, hamsters, and rhesus monkeys (1–4, 7). Unlike EMC and mengovirus, which grow well in L, HeLa, and KB cells, MM virus was reported not to reproduce and was lost as a mouse-pathogenic agent between the second and third passage in these cell lines (6). Growth in continuous cell lines (BHK-21 and McCoy cells) was recently shown by Pindak and Schmidt (*submitted for publication*). They found further that MM virus formed plaques in both BHK and L cells. Plaque formation by MM virus in L cells suggested that the virus, contrary to previous reports, propagated in this cell line. The objective of the present study was to determine the extent of MM virus replication in L cells.

MATERIALS AND METHODS

Cell cultures and medium. L (BBL) and BHK-21 cells were used for these studies. Both cell lines were grown in Blake-glass bottles with Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum. Monolayer cultures for experiments were prepared by adding 5×10^6 cells in 10 ml of MEM to 100-mm plastic dishes (Falcon Plastics) 24 hr prior to use. Tube cultures were prepared by seeding test tubes with approximately 10^6 cells in 1.5 ml of MEM 24 hr prior to use. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% CO_2.

Virus assay and propagation. MM virus (mouse brain suspension) was obtained from W. J. Kleinschmidt, Lilly Research Laboratories, Indianapolis, Ind. For plaque assay, the virus samples were diluted in Hanks balanced salt solution (HBS), and 0.5 ml of the appropriate dilution was added to each of two L-cell monolayer cultures. After a 1-hr adsorption period (37 C), the cultures were overlayered with 10 of MEM containing 1% agar, 1% fetal calf serum, and 0.08% protamine sulfate. The cultures were incubated for 48 hr and stained by adding 8 ml of an 0.02% solution of neutral red to each plate for 30 min. After removal of the stain, the plates were reincubated and the plaques were counted 4 to 6 hr later. For propagation of the virus in vitro, cell monolayer cultures were infected with 0.5 ml of undiluted stock virus which was allowed to adsorb for 1 hr. MEM (10 ml) plus 5% fetal calf serum was added to each plate and the cultures were incubated (37 C). The virus was harvested 24 hr later by two cycles of rapid freezing and thawing followed by low-speed centrifugation (2,500 × *g* for 5 min) to remove cellular debris. Propagation of the virus in vivo was done by injecting 0.2 ml of undiluted stock virus subcutaneously into mice. The brains were harvested just prior to death (3 to 4 days) and homogenized (2 ml of HBS per brain) with a tissue grinder. The brain suspension was then centrifuged at 2,500 × *g* for 20 min to remove cellular debris, and the fluid was collected.

RESULTS

The fact that MM virus produced plaques in L cells indicated that there was at least limited activity of the virus in this cell line. The following experiments were carried out to determine the extent of viral replication in this cell line.

Assay of viral replication by tissue destruction. Dilutions of BHK-21 cell-propagated MM virus were inoculated in 0.2-ml amounts into tube cultures of L cells containing 1.8 ml of MEM (12 tubes per dilution). Each dilution was similarly

TABLE 1. *Comparison of CPE in BHK-21 and L cells after inoculation with BHK-21-propagated MM virus*

Virus dilution	CPE		$TCID_{50}$[a] for BHK-21 cells
	L cells	BHK-21 cells	
Undiluted	3+	4+	10^7
10^{-1}	2+	4+	10^6
10^{-2}	2+	4+	10^5
10^{-3}	2+	4+	10^4
10^{-4}	+	4+	10^3
10^{-5}	0	4+	10^2
10^{-6}	0	4+	10^1
10^{-7}	0	4+	1
10^{-8}	0	0	0

[a] One $TCID_{50}$ is the amount of virus required to completely destroy (4+ CPE) a tube culture of BHK-21 cells.

TABLE 2. *Replication of MM virus in L cells without cytopathic effect*

Virus dilution	CPE in L cells (day)[a]				Virus content of L-cell fluids[b] (day)[a]			
	1	2	4	6	1	2	4	6
Undiluted	3+	3+	3+	3+	10^7	10^7	10^7	10^6
10^{-1}	2+	3+	3+	2+	10^8	10^7	10^7	10^7
10^{-2}	0	2+	+	2+	10^6	10^7	10^7	10^6
10^{-3}	0	+	+	2+	10^6	10^7	10^6	10^6
10^{-4}	0	+	+	0	10^5	10^6	10^6	10^5
10^{-5}	0	0	0	0	10^4	10^6	10^5	10^4
10^{-6}	0	0	0	0	0	10^4	1	0
10^{-7}	0	0	0	0	0	0	0	0
10^{-8}	0	0	0	0	0	0	0	0

[a] Day after infection.
[b] Titrations for virus yields were done on BHK-21 cells and expressed in $TCID_{50}$.

inoculated into four tube cultures of BHK-21 cells; these served as an indicator system for the amount of virus inoculated into the L-cell cultures. The cytopathic effect (CPE) obtained on the 6th day in each of the cell lines is given in Table 1. In L cells, there was no CPE in tubes inoculated with virus dilutions greater than 10^{-4}. In BHK-21 cells, on the other hand, complete CPE was obtained at a dilution of 10^{-7}; that inoculum was therefore considered to be equivalent to one tissue culture infective dose ($TCID_{50}$) of the virus. From these data it appears that the virus did not propagate in L cells and that the CPE was dependent on the size of inoculum (10^3 $TCID_{50}$ required to produce discernible CPE in L cells).

On the 1st, 2nd, 4th, and 6th days after inoculation, the culture fluids from L cells inoculated with each virus dilution were pooled and titrated for CPE in BHK-21 cells (free virus). The L cells in each tube were washed and 1 ml of MEM was added. After freezing and thawing twice, the cell suspensions were titrated as above (bound virus). Essentially all the virus was released by 24 hr, so that only free virus data are presented (Table 2). The maximal virus yield (10^2 to 10^3 $TCID_{50}$) was reached 1 to 2 days after infection. The absence of CPE in L cells at any particular time did not correlate with virus recovery. These data indicated that MM virus was replicating in L cells but was not producing CPE and, therefore, CPE in L cells could not be used as an indication of virus production.

Plaque-forming unit (PFU) analysis. To obtain more conclusive quantitative data, the following experiment was done. To each of four L-cell monolayer cultures (100-mm plastic dishes), 0.5 ml of MM virus (brain suspension; 8.1×10^7 PFU/ml) was added. After 1 hr for adsorption, the cultures were washed three times with HBS, and 10 ml of MEM plus 5% fetal calf serum was added to each plate. Two of the plates were immediately removed and the cultures were assayed for virus adsorption. At 24 hr after infection, the remaining two cultures were scored for CPE and assayed for PFU content. Fresh L-cell cultures

TABLE 3. *Propagation of MM virus in L cells*

L-cell passage	0 hr[a] (PFU/ml)	24 hr	
		PFU/ml	CPE
1	5.0×10^4	1.1×10^7	3+
2	2.8×10^4	1.1×10^7	0
3	1.4×10^4	1.5×10^6	0
4	1.6×10^4	1.7×10^6	0
5	1.5×10^4	1.3×10^6	0

[a] Virus attached to cells after a 1-hr adsorption period.

TABLE 4. *Propagation of MM(BHK_3)[a] virus in L cells*

L-cell passage	0 hr (PFU/ml)	24 hr	
		PFU/ml yield	CPE
1	1.2×10^5	4.7×10^8	4+
2	1.0×10^5	3.2×10^8	4+
3	1.1×10^5	1.3×10^8	4+
4	4.9×10^4	1.6×10^8	4+
5	1.2×10^5	2.0×10^8	4+
6	1.3×10^5	4.0×10^8	4+

[a] MM virus passed three times through BHK-21 cells.

were infected with undiluted virus from this first L-cell passage and the process was repeated. In this manner, the virus was passed five times in L cells. The results (Table 3) show that, through each of the five L-cell passages, MM virus was replicating and, with the exception of the first passage, there was no CPE. These data also show that its growth was very limited (less than 1 PFU/cell average yield).

After passage in BHK-21 cells, MM virus was observed to produce a limited amount of CPE in L cells (*see* Table 2), whereas no CPE was observed in L cells infected with MM virus not previously passed through BHK-21 cells. To determine whether passage of MM virus through BHK-21 cells altered its subsequent PFU yield from L cells, the following experiment was done. MM virus was passed three times in BHK-21 cells; it had a titer in L cells of 3.7×10^8 PFU/ml. This stock [henceforth designated MM(BHK)] was passed through L cells as described in the previous experiment. The results (Table 4) show that MM(BHK) virus replicated to a high titer with complete CPE in L cells. It is apparent from these data that passage of the MM virus through BHK cells resulted in its full adaptation to growth in L cells with an average yield of 200 to 300 PFU/cell.

Adaptation of MM virus to L cells. To determine the number of BHK-21 cell passages required for adaptation of MM virus to L cells, the following experiment was done. MM virus (brain suspension) was passed through L cells three times; it had a titer of 1.5×10^6 PFU/ml (Table 3). The virus was then passed through BHK-21 cells one, two, and three times. Each of these BHK-virus stocks was then passed through L cells as before. The results (Table 5) show that two passages through BHK-21 cells were sufficient to fully adapt the virus to L cells. Although one BHK-21 cell passage brought the virus titer up, it was not sufficient for full adaptation and further L-cell passages resulted in loss of titer and loss of CPE.

TABLE 5. *Number of BHK-21 cell passages required for L-cell adaptation of MM virus*

MML_3[a] BHK-21 passage	BHK-21 24-hr Virus yield (PFU/ml)	L-cell		
		Passage	24-hr Yield (PFU/ml)	24-hr CPE
1	4.0×10^7	1	4.7×10^7	1+
		2	2.6×10^7	2+
2	1.0×10^8	1	2.5×10^8	3+
		2	4.0×10^8	4+
3	1.3×10^8	1	1.3×10^8	3+
		2	3.5×10^8	4+

[a] MM virus passed through L cells three times (1.5×10^6 PFU/ml).

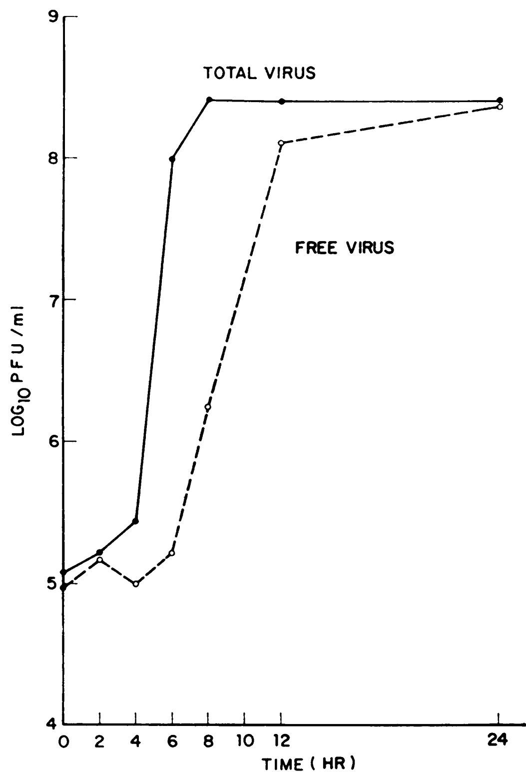

FIG. 1. *Replication of MM(BHK) virus in L cells. L-cell cultures were infected with MM(BHK) virus and assayed for total (●) and free (○) virus content at the indicated times.*

MM(BHK) virus growth curve in L cells. To study the kinetics of MM virus replication, replicate monolayer cultures of L cells (24 hr old; 10^7 cells per culture) were infected with 0.5 ml of MM(BHK) virus stock (4.7×10^8 PFU/ml). After 1 hr for adsorption (37 C), the cultures were washed three times with HBS, and 10 ml of MEM plus 5% fetal calf serum was added to each culture. At various times, samples were removed (fluid for free virus; cells and fluid for total virus) and assayed for PFU content. The results (Fig. 1) show that mature virus synthesis began at 4 hr and was completed by 8 hr. The virus was released slowly (less than 1% free at 8 hr) but was completely released by 24 hr.

DISCUSSION

We have shown that MM virus replicates in L cells. This finding is in direct contrast to that of Jungeblut and Kodza (6), who reported that L cells would not support the growth of MM virus.

These authors relied on CPE and on animal pathogenicity to determine viral replication. Had we used CPE as the criterion for viral replication, we also would have concluded erroneously that MM virus (mouse-brain stock) does not replicate in L cells. Passage of the mouse-brain MM virus stock through BHK-21 cells resulted in virus which replicated to high titers (10^8 PFU/ml) in L cells with full CPE. These new characteristics appear to be stable, since they are still present after 10 passages in L cells. At the present time we do not know the role of BHK-21 cells in fully "adapting" MM virus to good growth in L cells. Preliminary experiments, however, indicate that there is much more interferon present in L cell-produced MM virus stocks prepared with virus which has never been exposed to growth in BHK-21 cells than there is in those MM virus stocks with a prior exposure to growth in BHK-21 cells. We are currently investigating the role of interferon in the replication of MM virus in L cells.

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