Propagation of MM Virus in Continuous Cell Lines

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Baby hamster kidney (BHK), McCoy, and L cell lines were found to be suitable for isolation of MM virus from infected mouse brain tissue. The virus was recovered in high titer in the first passage in BHK and McCoy cells, with concomitant cytopathic effect (CPE). In L cells, virus yield was lower than in the other two cell lines and CPE was incomplete. Adaptation of the virus to BHK and McCoy cells by serial passages was evidenced by accelerated development of the CPE and increase in the virus titer. Plaques were obtained in all three cell lines when inoculated with infected mouse brain or with the tissue culture-propagated virus. In the BHK cells, the virus release preceded the appearance of CPE and maximal yield of virus was obtained after 1 to 3 days of incubation, depending on the size of inoculum. The BHK-propagated virus had the same lethality for mice as did the mouse brain-propagated stock, and there was no difference in the course of the disease caused by the two preparations.

The MM virus is highly pathogenic for the common laboratory animals, such as the mouse, hamster, and cotton rat, when administered by various extraneural routes (1, 6). It is stable under ordinary laboratory conditions (10) and is sensitive to interferon induced by statolon (8). Thus, it appears to be a very satisfactory representative of the encephalomyocarditis (EMC) group of viruses (2, 13, 14; J. Warren and J. E. Smadel, J. Bacteriol., p. 615–616, 1946) for a wide range of studies. It can be propagated in embryonated eggs (3) and in cultures of a variety of primary cells (4, 5, 11, 12). However, attempts to propagate it in HeLa, L, or KB cells were unsuccessful (7). The available literature does not indicate that it has been grown in serially propagated cell lines. For this reason it is normally propagated by mouse brain passage. This procedure is rather cumbersome, carries the inherent risk of inadvertent contamination of the stock virus with latent viruses, and lacks the flexibility required for various assays which can be accomplished with relative ease when continuous cell lines can be used.

This paper shows that some serially propagated cell lines are suitable for propagation of MM virus in high titer with concomitant production of cytopathic effect (CPE) and formation of plaques.

MATERIALS AND METHODS

Virus. Mouse brain suspension containing the MM virus was received through the courtesy of W. J. Kleinschmidt of the Lilly Research Laboratories, Indianapolis, Indiana. The virus was propagated in his laboratories by the mouse passage since 1947, when it was supplied by C. W. Jungeblut who with G. Dalldorf (6) made the original isolation. The infected brain tissue, diluted 1:100 in Hanks solution, was inoculated into 10 mice. The brains of five moribund and two dead mice were homogenized and resuspended in Hanks solution (2 ml per brain). After centrifugation, the pooled supernatant fluid was divided into small portions and stored at −60 C for future use.

Mice. Young adult male Swiss albino mice were inoculated subcutaneously with 0.2-ml amounts of suspensions of infected mouse brains or tissue culture fluids appropriately diluted in Hanks balanced salt solution. The animals were observed for illness or death daily for 14 days after infection.

Tissue cultures. The infected mouse brain suspension was inoculated into monolayers of baby hamster kidney (BHK) cells, into human synovial (McCoy) cells, and into mouse fibroblast (L_{929}) cells. The monolayers of cells were grown in test tubes in a 5% CO_2 atmosphere at 37 C. The growth medium was Eagle's basal medium supplemented with 10% fetal bovine serum. Each 1 ml of the medium contained 100 units of penicillin and 100 μg of streptomycin. For maintenance medium the serum content was decreased to 1%. This medium was dispensed in 1.8-ml amounts per tube. Usually four or more tubes were inoculated with 0.2 ml of the virus suspensions in any particular dilution. The tubes were observed daily for the presence of CPE for 4 to 8 days after inoculation. The extent of the CPE was graded as follows: 0, no CPE; +, isolated areas of cellular granulation or necrosis; 2+, granulation or necrosis in approximately 50% of the monolayer; 3+, granulation or necrosis in the entire

monolayer; and 4+, breakup of the monolayer. For plaque assays, the monolayers were grown in plastic petri dishes (Falcon) under 5% CO_2 atmosphere. The virus was allowed to adsorb for 45 min at 37 C. Subsequently, the monolayers were overlaid with 1% agar contained in the maintenance medium supplemented with protamine sulfate in 0.08% final concentration. After 48 hr of incubation at 37 C, the monolayers were stained with neutral red diluted 1:5000 in Hanks solution.

RESULTS

Propagation of MM virus in tissue culture. Insomuch as the original isolation of the MM virus was accomplished in the hamster (6) and primary cultures of embryonic hamster tissues were shown to support its growth (1), it was desirable to determine if the continuous line of BHK cells could also be used for its propagation. Decimal dilutions of the infected mouse brain tissue were inoculated into tubes of BHK cell monolayers. The tubes were examined daily for 6 days and the presence of the CPE was recorded. As Table 1 shows, the undiluted inoculum caused a definite CPE at 1 day after inoculation. Subsequently, the cytopathogenesis progressed to complete (4+) cell destruction in all tubes except in those inoculated with the 10^{-6} dilution. A similar effect was observed in McCoy cells inoculated with the same dilutions of the mouse brain stock virus, but the highest dilution of the inoculum producing CPE was 10^{-4}. In the L cells, the CPE was much less pronounced and was found only in the tubes which received the three highest concentrations of the inoculum.

At day 6 postinoculation, 0.2-ml amounts of the infected culture fluids from all three cell types were transferred into a parallel series of BHK cultures. Complete CPE developed during the ensuing 4 days in the tubes which were inoculated with the fluids from the L cell cultures containing the brain suspension in dilutions ranging from undiluted to 10^{-5} dilution. The fluids from BHK and McCoy cells also produced complete CPE to the same original dilution end point.

From the first BHK passage of the MM virus, three tubes inoculated with the 10^{-5} dilution of the infected mouse brain were frozen and thawed. Their contents were pooled and titrated in BHK cells. The highest dilution of this material giving CPE was 10^{-7}. In the ensuing four additional serial passages the titer varied from 10^{-6} to 10^{-7}. It was noticed in the third passage that the time required to produce complete CPE was shorter than in the first passage. Although 3 days were required for the undiluted mouse brain suspension to produce complete cell destruction (Table 1), in the third passage the cells were necrotized and the monolayers were disrupted at 1 day after inoculation in this and in the 10^{-1} dilution. The accelerated cell destruction was also noted in tubes inoculated from the higher dilutions of the virus. The development of the CPE appeared to be complete at day 4 postinoculation; the destruction of cells inoculated from the 10^{-7} dilution was complete and no CPE appeared beyond this dilution during an additional 2 days of incubation. Daily assays of the infected culture fluids revealed that the virus was released from the cells before the CPE could be detected and that with a relatively heavy inoculum (up to the 10^{-5} dilution) the maximal virus concentration in the culture was obtained at 1 day after inoculation. The propagation of the MM virus from our own mouse brain preparation in the BHK cells was repeated twice. It was also accomplished from the original material obtained from W. J. Kleinschmidt.

TABLE 1. *CPE[a] caused in three cell lines by the mouse brain passage of MM virus*

Inoculum dilution[b]	CPE on days after inoculation																	
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
	BHK cells						L cells						McCoy cells					
Undiluted	2+	3+	4+	4+	4+	4+	0	0	+	+	+	2+	2+	2+	3+	4+	4+	4+
10^{-1}	+	+	3+	4+	4+	4+	0	0	0	+	+	2+	0	+	2+	3+	4+	4+
10^{-2}	0	+	3+	4+	4+	4+	0	0	0	+	+	+	0	2+	3+	4+	4+	4+
10^{-3}	0	+	3+	4+	4+	4+	0	0	0	0	0	0	0	2+	3+	3+	4+	4+
10^{-4}	0	+	3+	4+	4+	4+	0	0	0	0	0	0	0	0	+	3+	3+	4+
10^{-5}	0	0	3+	4+	4+	4+	0	0	0	0	0	0	0	0	0	0	0	0

[a] Grading of the CPE: 0, no CPE; +, isolated areas of cellular granulation or necrosis; 2+, granulation or necrosis in approximately 50% of the monolayer; 3+, granulation or necrosis in the entire monolayer; and 4+, breakup of the monolayer.

[b] At 10^{-6} dilution, no CPE was observed for the three cell lines.

In an attempt to titrate the viral content of the BHK stock by the plaque assay method, we inoculated the virus onto monolayers of BHK, McCoy, and L cells grown in plastic petri dishes under a 5% CO_2 atmosphere. Of appropriate dilutions of the virus stock in Hanks solution, 0.4 ml were transferred into 6 to 8 plates of each cell line. Plaques were obtained in all three cell lines. The calculated average plaque-forming unit content per 1 ml of the BHK stock was as follows: 4.37×10^7 in BHK, 1.87×10^7 in McCoy, and 3.75×10^7 in L cells. The size of plaques in McCoy and L cells varied from pinpoint to approximately 3 mm in diameter. The larger plaques were predominant and their outlines were diffuse. Similar variation in the size of the plaques was also observed in the BHK cells, but the larger ones were 5 to 6 mm in diameter, with well-defined outlines. Plaques of the same type were also obtained when the BHK cells were inoculated with both the original and our mouse brain stock preparations of the virus.

Pathogenicity for mice of the BHK-propagated MM virus. The lethality of the third BHK passage of the virus was compared in mice with that of the mouse brain stock. Decimal dilutions of each preparation were made in Hanks solution. From dilutions of 10^{-4} to 10^{-8}, 0.2-ml amounts were inoculated into 10 mice. All animals were inspected daily for the next 15 days, and the deaths were recorded. The LD_{50} and mean survival time values for the group inoculated with the BHK-propagated virus were in close agreement with those obtained from mice infected with the mouse brain stock of the virus (Table 2).

Histopathological examination of the central nervous system of a moribund mouse infected with the 10^{-6} dilution of the BHK stock virus revealed periacute encephalitis present in all major areas of the brain, characterized by neu-

ronal necrosis, small to moderate infiltration of polymorphonuclear and mononuclear inflammatory cells. In some regions large focal areas of necrosis of the entire neurophil were present. The lesions were more frequently located in the gray matter but were also present to some extent in the white matter. The spinal cord was examined at the cervical, thoracic, and lumbar levels. Acute lesions, similar to those described for the brain, were found in the gray matter, but these were not as severe as in the brain.

DISCUSSION

The EMC group of viruses appears to affect wild rodents primarily, although isolated incidents of human infections have been reported (2, 13). Serologically the various members of the group appear to be strains of a single virus (14; J. Warren and J. E. Smadel, J. Bacteriol., p. 615–616, 1946). However, distinct differences in their adaptability to propagation in various cells in tissue culture have been reported. Thus, although EMC and mengovirus grew well in L, HeLa, and KB cells, replication of MM and Columbia SK viruses could not be achieved in them despite repeated attempts to adapt the viruses by serial blind passage of either culture fluids or of cells (7).

The MM virus has been propagated in high titer in the brains of mice, hamsters, and cotton rats (6) and in various tissue explants or primary cell cultures (4, 5, 11, 12). However, to minimize the possibility of contaminating the stock virus with latent viruses, passages in the intact animal or in primary cell cultures should be avoided whenever possible. We, therefore, explored the feasibility of adapting the MM virus to propagation in three cell lines. The virus was readily propagated in BHK and McCoy cells. A slight degree of adaptation was required in both cell lines, as evidenced by the slower development of the CPE in the first passage than in the later passages. Also, in the subsequent passages, the virus titer increased a hundredfold or more both in BHK and McCoy cells.

The behavior of the virus in L cells appeared to differ from that observed in BHK and McCoy cells. Whereas the cytopathogenicity study in this cell line was inconclusive (Table 1), the development of plaques indicated that there was at least limited virus activity. The results of additional studies (*unpublished data*) showed that, from infected mouse brain, the MM virus can be also propagated in L cells but with a lesser virus yield than in either BHK or McCoy cells.

The mortality data from simultaneous in vivo

TABLE 2. *Comparison of lethality for mice of mouse brain and BHK-propagated MM virus*

Virus dilution	Mouse brain			BHK culture		
	Mortality[a]	MST[b] (days)	LD50[c]	Mortality[a]	MST[b]	LD50[c]
	%			%		
10^{-4}	100	4.90		100	4.70	
10^{-5}	100	5.36		100	5.30	
10^{-6}	90	6.56	$10^{-6.24}$	90	6.33	$10^{-6.34}$
10^{-7}	40	7.75		20	8.00	
10^{-8}	0			10		

[a] Ten mice were tested.

[b] Mean survival time.

[c] Determined by the Reed and Muench formula (9).

titrations of the third passage of the virus in BHK cells and of the mouse brain stock have shown that the tissue culture-propagated stock had essentially the same lethality for mice as did the mouse brain-propagated stock (Table 2). The clinical symptoms after infection with the two stocks of the virus were indistinguishable and were in a good agreement with those described by Jungeblut and Dalldorf (6) who originally isolated the virus. The results of a histopathological examination of the central nervous system of a mouse infected with the BHK-propagated virus were fully compatible with the clinical findings.

ACKNOWLEDGMENTS

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