



AD712613

DDC
RECEIVED
OCT 15
A

Role of Interferon in the Propagation of MM Virus in L Cells

DAVID J. GIRON

Infectious Diseases Branch, Biosciences Division, USAF School of Aerospace Medicine, Brooks Air Force Base, Texas 78235

Received for publication 18 August 1969

MM virus propagated in mouse brain replicates to low titers in L cells without production of cytopathic effect (CPE). After growing the virus in BHK-21 cells, however, the virus replicates to high titers in L cells with complete CPE. It was found that suspensions of MM virus propagated in L cells directly from the mouse brain contained much more interferon than did suspensions of virus which had first been grown in BHK-21 cells. Mouse brain suspensions of the virus were also found to contain high interferon titers. Treatment of L cells with actinomycin D before infection with mouse brain-grown virus resulted in full virus replication with CPE. BHK-21 cell-grown virus diluted in L cell interferon behaved like mouse brain-grown virus in L cells. It is concluded that the presence of interferon in the inoculum is largely responsible for the suppression of MM virus replication in L cells.

MM virus was first isolated and described by Jungeblut and Dalldorf (3). It is classified with the encephalomyocarditis (EMC) group which includes Columbia SK, EMC, F, Mengo, and SVW (13). Members of this group appear to be immunologically indistinguishable strains of a single virus (12), but different strains have different growth requirements (4). Since its discovery, MM virus has been almost exclusively propagated by mouse brain passage because of the apparent failure of the virus to grow in continuous cell lines (4). Recently, however, propagation of MM virus in several continuous cell lines has been reported (1, 8). In L cells, mouse brain-grown MM virus replicated to relatively low titers [average yield of less than 1 plaque-forming units (PFU)/cell] without cytopathic effect (CPE). After growing the virus in BHK-21 cells, however, it replicated to high titers in L cells (200 to 300 PFU/cell) with complete CPE. In this report, we show that interferon is largely responsible for the suppression of MM virus growth in L cells.

MATERIALS AND METHODS

Cell cultures and medium. L and BHK-21 cells were grown on Blake-glass bottles in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum. Monolayer cultures were prepared by adding 5×10^6 cells in 10 ml of MEM to 100-mm plastic dishes (Falcon Plastics) 24 hr before use. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Viruses. MM virus (mouse brain suspension), obtained from W. J. Kleinschmidt, Lilly Research Laboratories, Indianapolis, Ind., and vesicular stomatitis virus (VSV) were used in these studies. MM virus was propagated in mouse brain, L cells (MML), and BHK-21 cells [MM(BHK)]. For production of virus stocks *in vitro*, cell monolayer cultures were infected with 0.5 ml of undiluted virus which was allowed to adsorb for 1 hr. A 10-ml amount of MEM plus 5% fetal calf serum was added to each plate, and the cultures were incubated at 37°C. The virus was harvested 24 hr later by two cycles of rapid freezing and thawing, followed by low-speed centrifugation ($2,500 \times g$ 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 Hanks balanced salt solution (HBS) per brain] with a tissue grinder. The brain suspension was then centrifuged at $2,500 \times g$ for 20 min to remove cellular debris, and the fluid was collected. VSV was propagated by inoculation of 10-day-old embryonated chicken eggs. The allantoic fluids were collected 24 to 48 hr after infection. Titration of the viruses was by the standard plaque assay method. For MM virus, the agar-MEM overlay was supplemented with 1% fetal calf serum and 0.08% protamine sulfate.

Interferon assay. Samples to be assayed for interferon activity were exposed to an ultraviolet (UV) lamp at a distance of 12 cm for 5 min. UV irradiation for this length of time completely inactivates MM virus and eliminates its capacity to induce antiviral inactivity in L cells (*unpublished data*). Twofold serial dilutions of each sample were made in MEM plus 5% fetal calf

serum. Cell cultures were treated with the various interferon dilutions (5 ml per culture) for 18 hr. Three of the most widely used methods for interferon titration were then employed: (i) inhibition of CPE, (ii) reduction of virus yield, and (iii) plaque inhibition (11).

RESULTS

Interference with MM virus infection. We have found a complete absence of CPE in L cells infected with MML virus; the infected cells remained completely viable. Thus, we had an ideal system with which to determine whether prior infection of L cells with MML virus interferes with MM(BHK) virus infection. Several L-cell monolayer cultures were infected with 0.5 ml of MML₃ virus stock (third L-cell passage; 1.5×10^6 PFU/ml). After 1 hr for adsorption, the cultures were washed three times and 10 ml of MEM plus 5% fetal calf serum was added to each culture. Identical cell cultures were treated in a like manner, except that they received HBS during the virus adsorption period. Twenty-four hours after infection, all of the cultures were washed three times and challenged with 0.5 ml of MM(BHK)L₂ virus

TABLE 1. Interference by MML₃ virus with the replication of MM(BHK)L₂ virus in L cells

Pre-infected with MML ₃ ^a	Virus titer (PFU/ml)		CPE	
	After adsorption (0 hr)	24-yr yield	24 hr	48 hr
Yes	8.0×10^4	8.7×10^4	None	None
No	2.0×10^5	3.0×10^8	4+	4+

^a L-cell cultures were infected with MML₃ virus, and control cultures remained uninfected. After 24 hr, all cultures were washed and infected with MM(BHK)L₂ virus.

TABLE 2. Induction of interferon activity by different MM virus stocks

Virus stock	Interferon activity (units/ml) ^a		
	100% CPE protection	50% Plaque reduction	50% Reduction in virus yield
Mouse brain	1,000	6,400	ND
MML ₂	256	2,560	512
MM(BHK)L ₂	4	ND	64

^a L-cell cultures were treated with dilutions of each interferon preparation for 18 hr. The cultures were then washed and infected with MM(BHK)L₁ virus (10 PFU/cell for CPE and virus yield assays; 100 PFU/plate for plaque reduction assay). Interferon activity titers are expressed as the reciprocal of the highest dilution giving the appropriate protection for each assay.

TABLE 3. Effect of trypsin treatment and centrifugation on the interferon activity of MML₂ in L cells

Challenge virus	Interferon titer ^a		
	Control	Trypsin treated ^b	Centrifuged ^c
MM(BHK)L ₁	640	80	640
VSV.....	640	80	640

^a Titers expressed as the reciprocal of the highest dilution giving 100% protection against CPE.

^b An 0.05-mg amount of trypsin/ml for 1 hr at 37 C.

^c Top portion of the supernatant fluid was collected after centrifugation at $170,000 \times g$ for 2 hr.

stock (4.7×10^8 PFU/ml). Unadsorbed virus was washed off, and 10 ml of MEM was added to each culture. One culture from each group was immediately assayed for virus content (0 hr). The remaining cultures were scored for CPE 24 and 48 hr after infection and assayed for 24-hr virus content. The results (Table 1) show that infection of L cells with MML₃ virus protected the cell cultures against infection with MM(BHK)L₂ virus.

Comparison of interferon production by MML and MM(BHK) viruses in L cells. Since the MML₃ virus inoculum in the above experiment was low (about 0.15 PFU/cell), one would expect that a maximum of 15% of the cells had been infected. The protection against MM(BHK)L₂ virus infection was therefore probably not due to prior infection of the cells. A more likely possibility was that interferon, either in the inoculum or released by the cells after infection, protected the cell cultures. The amount of interferon activity present in each of the stock MM virus preparations [mouse brain, MML₂, MM(BHK)L₂] was determined. Suspensions of each virus stock were irradiated with UV light for 5 min and assayed for interferon activity in L cells against MM(BHK)L₁ infection (see above). The results (Table 2) show that both the mouse brain and MML₂ virus stocks had high levels of interferon activity. MM(BHK)L₂ virus stock, on the other hand, had relatively little interferon activity. These data are consistent with the hypothesis that interferon is responsible for the suppression of MM virus replication in L cells. These data also show that, of the methods employed for interferon titration, plaque inhibition is the most sensitive. Complete protection against CPE, however, has proved to be a quick and reliable method for detecting the presence of interferon activity and was the method employed in the remaining experiments.

Identification of the viral inhibitor as an interferon. Before we could identify the inhibitor as

interferon, it was necessary to show that it met certain criteria used in identifying interferons, namely, that it was protein, nonsedimentable, not virus-specific, and that its protective action was inhibited by actinomycin D. MML₂ stock virus was irradiated with UV light for 5 min. Part of it was then treated with 0.05 mg of trypsin per ml for 1 hr (37 °C). Another portion was centrifuged (170,000 × g for 2 hr), and the upper portion of the supernatant fluid was collected. A third portion was used without further treatment. Twofold serial dilutions of each interferon preparation were made in MEM plus 5% fetal calf serum. Samples (2 ml) of each dilution were added to each of several L-cell monolayer cultures (60-mm plastic plates). After 18 hr of incubation, all of the cultures were washed three times; one-half of them were infected with MM(BHK)L₁ stock virus, and the other half were infected with VSV in the usual manner (10 PFU/cell). The cultures were scored for CPE 24 hr after infection. The results (Table 3) show that the antiviral activity was sensitive to trypsin, was not removed by centrifugation, and was not virus-specific.

We next determined the effect of prior treatment with actinomycin D on the action of the interferon preparation. Monolayer cultures of L cells were divided into six groups. Group 1 was treated with interferon (64 protective units) for 4 hr, group 2 was treated with actinomycin D for 1 hr (5 μg/ml) followed by interferon for 4 hr, groups 3 and 6 were treated with actinomycin D only, and groups 4 and 5 were not treated. All of the cultures were then washed three times, and the first four groups were infected with MM(BHK)L₁ (10 PFU/cell). Groups 5 and 6 were cell controls and were not infected. The cultures were scored for CPE and were assayed for virus content 24 hr after infection. The results (Table 4) show that prior treatment of cells with actinomycin D inhibited the action of the interferon preparation. From the results of the above experiments, we conclude that the antiviral agent was an interferon.

TABLE 4. Effect of prior treatment with actinomycin D on interferon action

Culture treatment	CPE (24 hr)	Virus yield (PFU/ml)
Interferon, ^a 4 hr	None	9.1 × 10 ⁶
Actinomycin D 1 hr; interferon 4 hr	4+	2.7 × 10 ⁶
Actinomycin D, 1 hr	4+	3.3 × 10 ⁶
None (virus control)	4+	2.7 × 10 ⁶
None (cell control)	None	
Actinomycin D, 1 hr (cell control)	None	

^a A total of 64 CPE protective units.

TABLE 5. Effect of actinomycin D on MML₄ virus replication in L cells

Treated with actinomycin D ^b	CPE		Virus (PFU/ml)	
	24 hr	48 hr	24 hr ^c	48 hr
Yes	±		10 ⁸	
Yes	±	4+		2.5 × 10 ⁷
No	None		1.5 × 10 ⁵	
No	None	None		9.8 × 10 ³

^a Mouse brain-propagated MM virus passed four times in L cells.

^b Amount was 5 μg/ml for 1 hr before infection.

^c Cells were frozen and thawed twice to release virus.

Effect of actinomycin D on MM virus replication in L cells. The well-known finding that cellular ribonucleic acid synthesis is required for the production or inhibitory action of interferon (2, 5-7, 9, 10), or for both, enabled us, in the following experiment, to determine more conclusively the extent of interferon involvement in the suppression of MM virus replication. Two 24-hr, L-cell monolayer cultures were treated with actinomycin D for 1 hr (5 μg/ml). Two additional cultures served as controls and were not treated. All of the cultures were then washed twice and infected with 0.5 ml of MML₄ (1.7 × 10⁶ PFU/ml). After a 1-hr adsorption period, the cultures were washed three times, and 10 ml of MEM plus 5% fetal calf serum was added to each culture. At 24 and 48 hr after infection, the cultures were scored for CPE and assayed for virus content. The results (Table 5) show that actinomycin D treatment resulted in 24-hr PFU titers which were about 2.5 logs higher than those of control cultures. The PFU titer was almost equal to that of MM(BHK) virus stocks. On subsequent L-cell passages, this virus stock behaved like MM(BHK) virus with average yields of 200 to 300 PFU/cell and production of complete CPE. From these data, we conclude that interferon was responsible for the failure of MM virus to grow in L cells. These data also show that, by 48 hr after infection (24 hr after maximal virus yield), the PFU titers of both the treated and control cultures had dropped, suggesting that MM virus is sensitive to prolonged exposure at a temperature of 37 °C.

Effect of added interferon on the replication of MM(BHK) in L cells. The interferon present in MM virus grown in mouse brain or L cells (Table 2), rather than interferon produced de novo, was postulated to be the protective factor. We reasoned that if L-cell cultures were infected with MM(BHK) containing no L-cell interferon, but

TABLE 6. Effect of interferon in the inoculum on the replication of MM(BHK)L₂ virus in L cells

Diluting fluid	MM(BHK)L ₂ inoculum (PFU/culture)	CPE		Virus yield (PFU/culture)	
		24 hr	48 hr	24 hr	48 hr
None.....	2.4×10^8	4+	4+	2.4×10^9	7.8×10^8
HBS.....	2.4×10^7	4+	4+	3.2×10^9	8.8×10^8
Interferon ^a	2.4×10^7	4+	4+	2.7×10^8	1.0×10^8
HBS.....	2.4×10^6	3+	3+	2.3×10^9	1.5×10^9
Interferon.....	2.4×10^6	None	None	1.2×10^7	4.0×10^6
HBS.....	2.4×10^5	2+	3+	1.7×10^9	2.0×10^9
Interferon.....	2.4×10^5	None	None	$<10^3$	$<10^2$

^a Interferon prepared from MML₂ stock virus. Each MM(BHK)L₂ inoculum contained 256 CPE protective units.

diluted in HBS to contain PFU titers similar to those of MML, full yields of virus should be obtained. If MM(BHK) were diluted in L-cell interferon, however, virus yields should be reduced and should, in fact, be similar to those from cultures infected with MML virus. To test this hypothesis, 10-fold dilutions of MM(BHK)L₂ virus stock (4.7×10^8 PFU/ml) were made in L-cell interferon (256 protective units/ml). For control, similar dilutions of the virus were made in HBS. L-cell monolayer cultures were infected in replicates of four with each virus concentration. Cultures were examined for CPE and assayed for virus content at 24 and 48 hr after infection. The results (Table 6) show a yield of 2.4×10^9 PFU/culture, with complete CPE from cultures infected with undiluted MM(BHK)L₂. Virus yields from cultures infected with virus diluted in HBS were similar to those of the control groups, although the CPE was not always complete. Virus yields from cultures inoculated with virus diluted in interferon, however, were different from those of control cultures. A 1:10 dilution of the virus reduced the yield by 90% (2.7×10^8 PFU/culture). A 1:100 dilution in interferon reduced the yield by more than 99% (1.2×10^7 PFU/culture) with no production of CPE. This dilution of MM(BHK)-L₂ virus contained approximately the same number of PFU as the MML virus stock. There was essentially no virus production from cultures infected with MM(BHK)L₂ virus diluted 1:1,000 in interferon. These results indicate that the L-cell interferon present in the inoculum plays a major role in preventing CPE and in reducing viral replication in L-cell cultures infected with MML virus.

DISCUSSION

MM virus replicated to high titers with production of CPE in L cells if it was first passed through BHK-21 cells (1). Before BHK-21 cell passage,

the virus did not produce CPE in L cells and the viral replication was greatly reduced. The data in the present study indicate that interferon present in the inoculum is primarily responsible for the suppression of MM virus growth in L cells. The possibility that interferon formed *de novo* after viral infection is also involved cannot be ruled out at this time. From the data, we suggest that the suppression of MM virus replication in L cells occurred in the following manner: MM virus (mouse brain or L cell produced) contained high concentrations of interferon (Table 2) and relatively low PFU titers. The multiplicity of infection with these virus stocks was low (usually less than 1 PFU/cell), resulting in the infection of only a small percentage of the cells. The rest of the culture was in effect treated with a high concentration of interferon for 1 hr during the virus adsorption period and was protected against infection with newly synthesized virus. The virus yield was thus limited, and the culture was protected against CPE.

MM virus appears to be unusually sensitive to interferon, and we are currently investigating the possibility that the MM virus-L cell system may provide an improved assay for mouse and L-cell interferons.

ACKNOWLEDGMENTS

I thank P. T. Allen, J. P. Schmidt, and F. F. Pindak for helpful discussions and R. Ibarra for excellent technical assistance.

LITERATURE CITED

1. Giron, D. J., and F. F. Pindak. 1969. Propagation of MM virus in L cells. *Appl. Microbiol.* 17:811-814.
2. Ho, M. 1964. Identification and "induction" of interferon. *Bacteriol. Rev.* 28:367-381.
3. Jungeblut, C. W., and G. Dalldorf. 1943. Epidemiological and experimental observations on the possible significance of rodents in a suburban epidemic of poliomyelitis. *Amer. J. Public Health Nat. Health* 33:169-172.
4. Jungeblut, C. W., and H. Kodza. 1967. Studies on propagation of the Col. SK group of viruses in various tissue culture media. *Proc. Soc. Exp. Biol. Med.* 96:133-139.

5. Levine, S. 1964. Effect of actinomycin D and puromycin dihydrochloride on action of interferon. *Virology* 24: 586-588.
6. Levy, H. B., D. Axelrod, and S. Baron. 1965. Messenger RNA for interferon production. *Proc. Soc. Exp. Biol. Med.* 118: 384-385.
7. Lockart, R. Z. 1964. The necessity for cellular RNA and protein synthesis for viral inhibition resulting from interferon. *Biochem. Biophys. Res. Commun.* 15:513-518.
8. Pindak, F. F., and J. P. Schmidt. 1969. Propagation of MM virus in continuous cell lines. *Appl. Microbiol.* 17:815-818.
9. Taylor, J. 1964. Inhibition of interferon action by actinomycin. *Biochem. Biophys. Res. Commun.* 14:447-451.
10. Wagner, R. R. 1964. Inhibition of interferon biosynthesis by actinomycin D. *Nature (London)* 204:49-51.
11. Wagner, R. R., A. H. Levy, and T. J. Smith. 1968. Techniques for the study of interferons in animals virus-cell systems, p. 1-52. *In* K. Maramorosch and H. Koprowski (ed.), *Methods in virology*, vol. 4. Academic Press Inc., New York.
12. Warren, J. 1959. Infections of minor importance, p. 896-924. *In* T. M. Rivers and F. L. Horsfall (ed.), *Viral and rickettsial infections of man*, 3rd ed. J. P. Lippincott Co., Philadelphia.
13. Wilner, B. T. 1969. A classification of the major group of human and other animal viruses, 4th ed. Burgess Publishing Co., Minneapolis.

Unclassified

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) USAF School of Aerospace Medicine Aerospace Medical Division (AFSC) Brooks Air Force Base, Texas		2a. REPORT SECURITY CLASSIFICATION Unclassified	
		2b. GROUP	
3. REPORT TITLE ROLE OF INTERFERON IN THE PROPAGATION OF MM VIRUS IN L CELLS			
4. DESCRIPTIVE NOTES (Type of report and inclusive dates)			
5. AUTHOR(S) (First name, middle initial, last name) David J. Giron			
6. REPORT DATE March 1969		7a. TOTAL NO. OF PAGES 5	7b. NO. OF REFS 13
8a. CONTRACT OR GRANT NO.		9a. ORIGINATOR'S REPORT NUMBER(S) SAM-TR-70-256	
8b. PROJECT NO. c. Task No. 775308		9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	
10. DISTRIBUTION STATEMENT This document has been approved for public release and sale; its distribution is unlimited.			
11. SUPPLEMENTARY NOTES Appl. Microbiol. 18(4):584-588 (1969)		12. SPONSORING MILITARY ACTIVITY USAF School of Aerospace Medicine Aerospace Medical Division (AFSC) Brooks Air Force Base, Texas	
13. ABSTRACT MM virus propagated in mouse brain replicates to low titers in L cells without production of cytopathic effect (CPE). After growing the virus in BHK-21 cells, however, the virus replicates to high titers in L cells with complete CPE. It was found that suspensions of MM virus propagated in L cells directly from the mouse brain contained much more interferon than did suspensions of virus which had first been grown in BHK-21 cells. Mouse brain suspensions of the virus were also found to contain high interferon titers. Treatment of L cells with actinomycin D before infection with mouse brain-grown virus resulted in full virus replication with CPE. BHK-21 cell-grown virus diluted in L cell interferon behaved like mouse brain-grown virus in L cells. It is concluded that the presence of interferon in the inoculum is largely responsible for the suppression of MM virus replication in L cells.			

DD FORM 1 NOV 65 1473

Unclassified

Security Classification

Unclassified

Security Classification

14	KEY WORDS	LINK A		LINK B		LINK C	
		ROLE	WT	ROLE	WT	ROLE	WT
	Microbiology Interferon MM virus Virology						

Unclassified

Security Classification