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Interferon-Inducing Characteristics of MM Virus

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Interferon induction by MM virus in mice and in L cells was studied. In mice the virus readily induced interferon. The time of appearance was dose-dependent. A large virus dose induced interferon by 4 hr, whereas a small dose resulted in interferon production which paralleled virus replication 24 hr after infection. In L cells the interferon-inducing capacity of the virus was rapidly destroyed by ultraviolet light irradiation. Heating (56 C) of the virus, on the other hand, greatly increased its ability to induce interferon. Interferon production could also be increased by prior treatment of the cells with homologous interferon (priming). The increase in interferon production after priming was dependent on the concentration of interferon used for priming, the length of interferon treatment, and the multiplicity of infection. It is suggested that MM virus might be useful for the further study of the mechanisms involved in the production and action of interferon.

MM virus (34), a potent inducer of interferon, is highly virulent for mice and quite sensitive to the protective action of interferon in cell cultures (1, 8) and mice (Giron et al., *submitted for publication*). Because these attributes suggested that the virus was well suited for interferon studies, we examined in some detail its ability to induce interferon in vivo and in vitro under various experimental conditions. These conditions included pretreating cultures with interferon, heating the virus, exposing it to ultraviolet (UV) light, and varying the multiplicity of infection or dose of virus injected.

MATERIALS AND METHODS

Virus, cell cultures, and animals. A stock suspension of MM virus containing 2.7×10^8 plaque-forming units (PFU) per ml was used in the in vitro experiments. It was prepared and assayed in L-cell monolayer cultures as described previously (8). Other virus preparations were propagated in L cells or BHK-21 cells as indicated in the text. All virus dilutions were made in Hanks balanced salt solution (HBS). For injection into mice, the virus was diluted to contain the desired number of PFU in 0.2 ml of HBS. The mice used were male Swiss albinos weighing 14 to 20 g. L cells were grown in Blake glass bottles in a modified Eagle's medium containing HBS, twice the prescribed concentration of amino acids and vitamins, and 10% fetal calf serum (6). Monolayer cultures were prepared by adding the desired concentration of cells in 10 ml of medium to 100-mm plastic dishes (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.), or in 5-ml to 60-mm dishes 24 hr prior to use. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% CO_2.

Heat treatment. Two-milliliter samples of the virus stock suspension were dispensed into thin-walled glass vials and kept in ice water until heated. At specified intervals the vials were immersed in a water bath set at 56 C. The vials were shaken periodically, and at the end of the treatment period all were removed simultaneously and transferred to ice water with shaking to equilibrate the temperature.

UV irradiation. Five milliliters of the virus stock suspension was added to each of several 100-mm plastic dishes. Samples were exposed, for various lengths of time with constant shaking, to a Westinghouse G8T15 UV lamp at a distance of 12 cm (8×10^3 ergs per cm^2 per sec). Samples were immersed in ice water immediately after irradiation.

Interferon production and assay. Duplicate L-cell monolayer cultures (100-mm dishes) were inoculated with 0.5 ml of the virus sample to be tested for interferon-inducing capacity. After a 1-hr adsorption period at 37 C, the cultures were washed once with HBS and nourished with 10 ml of medium. Fluids were collected and pooled 48 hr after virus inoculation and assayed for interferon. Preparation of samples to be assayed for interferon activity was described previously (8). Two methods for interferon titration were employed: (i) 50% plaque reduction (PR_{50}; reference 33) and (ii) 50% inhibition of viral nucleic acid synthesis ($INAS_{50}$) which was developed during the course of these studies. Both methods employed MM virus as the challenge virus. The $INAS_{50}$ method was used in the later experiments of this study because of its advantage of reliability, speed, and convenience (1). Using the criteria outlined previously (8), the viral inhibitor was identified as an interferon.

RESULTS

Interferon induction in mice. In several virus-host systems virulence was inversely related to

interferon production, to the sensitivity of the virus to interferon's protective action (10, 23–25), or both. However, the correlation has not been universally observed (4, 19, 20, 31). The relationship between lethal MM virus infection and interferon production was examined in two experiments with mice. In both experiments the mice were given enough virus (propagated in BHK-21 cells) to cause 100% mortality. Table 1 shows the occurrence of virus and interferon in the sera and brains of mice inoculated intraperitoneally with 8×10^4 PFU [~2,000 median lethal dose (LD_{50}).] At intervals after virus injection, mice were sacrificed in groups of 10 and the sera and brains collected. The brains were pooled and suspended in HBS (2 ml/brain) with a Dounce homogenizer. Particulate material was removed by centrifugation. The pooled sera and brain extracts were assayed for virus and interferon content. There was increased viremia 24 hr after virus inoculation with a concomitant rise in the interferon titer. By 72 hr the virus was no longer detectable in the serum and relatively little interferon remained. Virus and interferon appeared in the brain 24 hr postinfection, and increased titers were observed through 96 hr (a few hours before death). Interferon production parelleled viral replication, but it was not effective in protecting the animal against lethal infection.

Table 2 shows the results obtained when the virus dose was 8×10^7 PFU/mouse. Sera and brains were collected at intervals and assayed for interferon and virus content as described above. By 15 min postinfection the serum-virus titer had become relatively stable at about 10^5 PFU/ml until at least 7 hr. By 24 hr the titer had dropped to 2.3×10^3 PFU/ml, and by 48 hr no virus could be detected. These results contrast with those of the previous experiment (Table 1) which showed a rise in serum-virus titer at 24 hr. Interferon was first detected in the serum at 4 hr; the titer increased until at least 7 hr and then was decreased by 24 hr. This pattern was similar to those reported in mice injected with large doses of other viruses (2, 27, 35). These data suggest that the early induction of interferon might have prevented the early replication of MM virus seen in the previous experiment. This interferon did not, however, prevent the virus from reaching the brain where it was detectable within 30 min after virus inoculation and where it replicated rapidly. Interferon production in the brain again appears to reflect viral replication.

TABLE 1. *Replication of MM virus and interferon production in mice after a low virus inoculum*

Time after virus inoculation[a] (hr)	Virus titer (PFU/ml)[b]		Interferon titer (units/ml)[b]	
	Serum	Brain	Serum	Brain
0	0	0	<4	<4
4	1.7×10^3	0	<4	<4
7	3.3×10^3	0	<4	<4
24	1.9×10^5	1.1×10^3	4,352	200
48	1.0×10^4	2.0×10^5	1,800	300
72	0	1.1×10^7	300	4,400
96	0	6.1×10^7	<4	6,600

[a] Intraperitoneally, 8×10^4 PFU were injected.
[b] Interferon and virus titers based on pooled samples from 10 mice at each time period. Interferon titers are expressed as the reciprocal of the highest dilution to inhibit MM virus plaque formation by 50%.

TABLE 2. *Replication of MM virus and interferon production in mice after a high virus inoculum*

Time after virus inoculation[a] (hr)	Virus titer (PFU/ml)[b]		Interferon titer (units/ml)[b]	
	Serum	Brain	Serum	Brain
5[c]	2.3×10^6	0	<10	<10
15[c]	7.5×10^4	0	<10	<10
30[c]	5.0×10^4	40	<10	<10
1	9.0×10^4	10	<10	<10
2	1.9×10^5	40	<10	<10
4	8.5×10^4	1.0×10^3	160	<10
7	1.0×10^5	7.6×10^3	3,060	<10
24	2.5×10^3	1.0×10^6	640	2,000
48	0	2.4×10^7	<10	4,000

[a] Intraperitoneally, 8×10^7 PFU were injected.
[b] Interferon and virus titers based on pooled samples from 10 mice at each time period. Interferon titers are expressed as the reciprocal of the highest dilution to inhibit MM virus plaque formation by 50%.
[c] Expressed as minutes.

Interferon induction by UV-irradiated or heated virus. Wide variabilities in interferon production have been found after treatment of viruses with UV or thermal energy. In some systems interferon production occurs in the absence of virus replication, whereas in others it is closely correlated with virus production (12, 32). The effect of heat and UV light on the interferon-inducing capacity of MM virus in L cells was investigated.

UV irradiation of some viruses for short periods of time has led to increased interferon yields (3, 12, 15, 22, 35), whereas long periods of irradiation resulted in loss of interferon-inducing capacity. MM virus was irradiated for varying periods, and a portion of each sample was assayed for infectious virus. The remaining portion was tested for its capacity to induce interferon (*see* above). The results showed that infectivity and interferon-

inducing capacity were quite sensitive to UV energy. Exposure for 5 sec (4×10^4 ergs/cm^2) resulted in less than 10^{-3} survival of the virus with a slight rise in interferon production (1,000 $INAS_{50}$ units by control versus 3,000 units by inactivated virus). Exposure for 15 sec (1.2×10^5 ergs/cm^2) reduced interferon production to 30 units with less than 10^{-4} survival of the virus. Irradiation for 30 sec (2.4×10^5 ergs/cm^2) or more reduced both the infective virus and the interferon-inducing capacity to below detectable levels.

Like UV irradiation, heat treatment of viruses has been found to have differing effects on interferon induction, depending on the host-virus systems studied or the experimental methods employed (16, 22, 30, 35). The kinetics of interferon induction by heated MM virus were studied using three stocks of virus which had undergone at least three serial passages in L cells and contained more than 2,000 $INAS_{50}$ units of interferon. The results obtained with one of the virus suspensions are shown in Fig. 1 and are similar to those obtained with the other virus stocks tested. The virus titer was reduced to 10^{-3} to 10^{-4} survival after 30 min of heating. The remaining virus was stable to heat for at least 2 hr. Five minutes of heating increased the interferon-inducing activity twofold; with continued heating the activity diminished and then again increased. Interferon production appeared to be correlated to virus production (progeny virus). Figure 2 shows the results obtained using a preparation of virus which had been propagated only once in L cells and contained only small amounts of interferon (<70 $INAS_{50}$ units). The virus was rapidly inactivated by heat, the thermostable fraction was much smaller, and the effect of thermal exposure on the interferon-inducing activity was quite different from that in the previous experiments (Fig. 1). Five minutes of exposure had no significant effect on interferon induction. By 30 min, however, the interferon-inducing activity increased about 11-fold and remained relatively stable with increased exposure.

The effect of prolonged heating on the interferon-inducing activity of MM virus was also investigated. Four preparations of the virus were heated at 56 C for 1 and 18 hr. The samples were then tested for residual interferon and virus content. L-cell monolayer cultures were inoculated with the heated material, and samples were collected at 24 and 48 hr after inoculation. The samples were assayed for interferon and virus content (Table 3). The most significant results seen were the unexpectedly large increases in interferon-inducing activity which were associated with prolonged heating of three of the virus batches. Heating the virus for 18 hr led to interferon yields as much as 250 times greater than those induced by the unheated virus controls. The inducing activity of virus batch A was completely destroyed by heating for 18 hr. The complete destruction of its infectivity probably accounts for the absence of interferon induction. As in the previous experiments a correlation was apparent between interferon production and virus replication. Although replication of the virus to a high titer was not invariably associated with a high interferon titer, high interferon titers were never found unless the virus titer was $> 10^6$ PFU/ml at the time of sampling. The data shown in Fig. 1 and 2 suggest that the increased inducing activity was correlated with the inactivation of interferon in the inducing material. A similar correlation is suggested with virus batches A, B, and C. Batch D, however, was prepared in BHK-21 cell cultures and contained no detectable interferon. It is probable, therefore, that the elimination of interferon from the virus preparations was not related to the increased

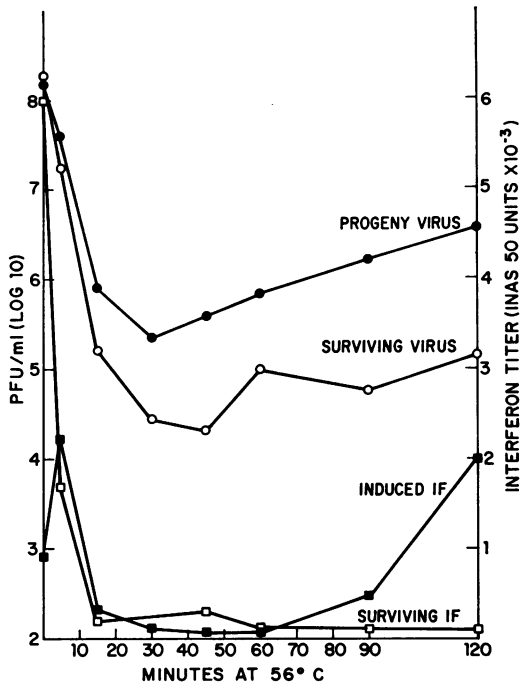

FIG. 1. *Interferon (IF) induction and virus replication by heated virus. MM virus was propagated in L cells for five serial passages. The virus suspension was heated at 56 C for the times indicated. Heated samples were assayed for surviving virus and interferon content. Their ability to induce interferon and propagate virus was determined. Samples for the assay of induced interferon and progeny virus were collected 48 hr after infection of L-cell monolayer cultures with the heated material.*

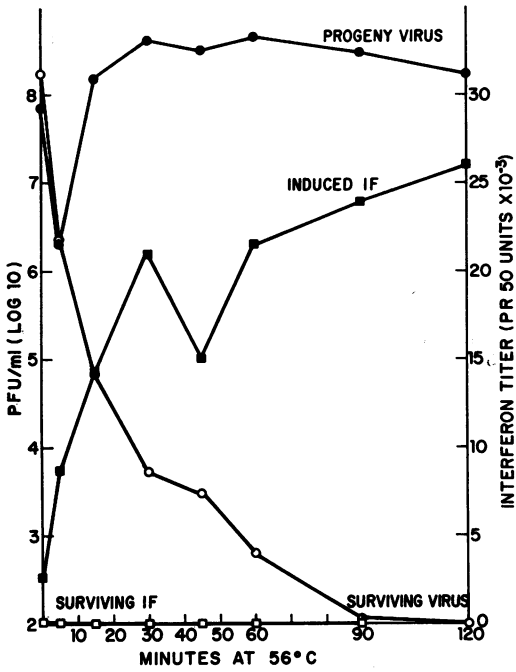

FIG. 2. *Details are the same as for Fig. 1 except that the virus suspension used had undergone only one L-cell passage.*

activity. Preliminary experiments (*data not shown*) indicate that simple dilution of the virus to titers similar to those found after heating does not result in the marked increase in interferon titers seen in Table 3.

Interferon production by interferon-treated cultures. Pretreatment with homologous interferon has been reported to increase or suppress interferon production by infected cultures, the effect apparently varying inversely with the amount of interferon used for pretreatment (7, 18, 21, 28). We examined the effects of pretreating L-cell cultures with concentrations of interferon for four time-intervals on subsequent interferon production induced by MM virus and on virus replication. In a typical experiment (Table 4), monolayer cultures containing 10^7 cells each were treated with interferon at concentrations of 0, 30, 600, or 3,000 $INAS_{50}$ units. At various intervals fluids were removed and the cultures were washed with HBS and challenged with 10 PFU of MM virus per cell. The culture fluids were collected after an additional 48 hr and assayed for interferon activity and virus titer. Treatment for 1 hr with 30 or 600 units of interferon had no significant effect on virus replication or interferon production. However, pretreatment with 3,000 units for 1 hr resulted in a fivefold increase in interferon

TABLE 3. *Increased interferon-inducing activity of heated MM virus*

Virus batch[a]	Time at 56 C (hr)	Interferon titer[b]			Virus titer (PFU/ml)			CPE[c] at 48 hr
		Residual	Induced		Residual	Progeny		
			24 hr	48 hr		24 hr	48 hr	
A	0	202	4,400	4,300	4.9×10^8	1.5×10^8	1.3×10^7	3+
	1	7	3,700	9,700	2.0×10^5	1.4×10^8	3.4×10^7	4+
	18	<3	<4	<2	<10	<10	<10	—
B	0	4,400	2,550	1,300	1.2×10^8	2.2×10^8	8.0×10^6	3+
	1	265	580	3,850	2.6×10^4	2.0×10^6	2.8×10^6	1+
	18	35	<60	27,000	2.0×10^2	1.3×10^4	1.9×10^8	1+
C	0	5,000	420	480	2.6×10^8	1.2×10^8	5.1×10^7	4+
	1	178	6,800	11,000	2.4×10^5	2.8×10^7	7.0×10^6	3+
	18	4	<100	34,000	2.0×10^2	2.6×10^5	4.5×10^7	2+
D	0	<3	205	225	9.0×10^7	2.2×10^8	5.6×10^7	4+
	1	<3	1,050	4,400	1.6×10^5	1.5×10^8	1.0×10^7	4+
	18	<3	<60	54,000	2.5×10^1	7.5×10^4	1.9×10^8	3+

[a] Batches A, B, and C were passed in L cells 1, 3, and 5 times, respectively; batch D was passed three times in L cells, then once in BHK-21 cells.

[b] Interferon was assayed by the plaque reduction technique using MM virus as the challenge agent. The titer is expressed as the reciprocal of the interferon dilution causing a 50% reduction in plaque number.

[c] Cytopathic effect.

TABLE 4. *Interferon and virus yields from cultures pretreated with different concentrations of interferon for different lengths of time*[a]

Concn of interferon used for pretreatment ($INAS_{50}$ units)	Length of pretreatment with interferon							
	1 hr		4 hr		6 hr		24 hr	
	Interferon yield	Virus yield	Interferon yield	Virus yield	Interferon yield	Virus yield	Interferon yield	Virus yield
0	2,400	2.5×10^7	2,000	2.2×10^7	1,900	2.8×10^7	2,400	4.5×10^7
30	3,000	3.4×10^7	10,500	3.4×10^6	8,800	4.8×10^5	10,300	3.9×10^5
600	2,300	2.0×10^7	9,700	1.5×10^5	11,200	8.6×10^4	14,500	6.2×10^4
3,000	12,600	1.9×10^7	10,000	1.4×10^5	8,500	8.9×10^4	1,400	1.0×10^5

[a] Each culture contained 10^7 L cells. After treatment for the period indicated, the interferon was removed, and the cultures were washed and inoculated with 10 PFU of MM virus per cell. Interferon and virus present in the culture fluids 48 hr postinoculation were assayed in L cells. Interferon was assayed by the 50% inhibition of viral nucleic acid synthesis ($INAS_{50}$) technique. All virus titers are given in PFU/ml.

production without significantly affecting virus replication. Pretreatment for 4 or 6 hr with any of the interferon concentrations had about the same stimulating effect on interferon yields (four- to sixfold), though the effect on virus yield varied. Pretreatment for 24 hr with any of the three interferon concentrations inhibited virus yield to a similar degree, but interferon production varied. The two lower concentrations increased interferon yields four- and sixfold, whereas 3,000 units decreased the interferon yield to about half of control cultures. Unlike the results of the previous studies, interferon production in interferon-treated cultures appeared to be unrelated to virus replication.

The role of multiplicity of infection in the production of interferon by interferon-treated cells was also examined. L-cell cultures were treated with 3,000 units of interferon for 4 hr. Control cultures were treated with medium. All cultures were washed and then inoculated at multiplicities of infection of 10, 0.01, or 0.0001 PFU per cell. After 48 hr the culture fluids were collected and assayed for interferon (Table 5). In control cultures multiplicities of 10 and 0.01 each produced 1,600 units of interferon, whereas a multiplicity of 0.0001 induced only 330 units. At each multiplicity the production of interferon was significantly altered by pretreating the cultures with interferon. At 10 PFU per cell interferon production was increased about 900%, although at multiplicities 0.01 and 0.0001 PFU/cell, pretreatment inhibited production by 90% or more. These data demonstrate that the effect of interferon pretreatment on subsequent interferon production was dependent not only on the amount of interferon used and the length of pretreatment but also on the multiplicity of infection of the inducer virus.

TABLE 5. *Interferon production by primed cells after inoculation with different doses of virus*

Primed with interferon[a]	Virus inoculum (PFU/10^6 cells)	CPE[b] (48 hr)	48-hr interferon yield ($INAS_{50}$ units)[c]
No	10^7	4+	1,600
Yes	10^7	1+	15,000
No	10^4	4+	1,600
Yes	10^4	3+	120
No	10^2	4+	330
Yes	10^2	0	33

[a] Cells treated with 3,000 units of interferon for 4 hr prior to infection.

[b] Cytopathic effect.

[c] Fifty per cent inhibition of viral nucleic acid synthesis.

DISCUSSION

MM virus has proven to be useful for studies involving interferon in mice (14). This virus is virulent for mice yet is sensitive to interferon and is a potent inducer of interferon in mice and L-cell cultures (8). The purpose of these studies was to examine some aspects of the induction of interferon by this virus.

The kinetics of interferon production in mice varied with the concentration of virus in the inoculum. Interferon was first detected in the serum 4 hr after intraperitoneal inoculation with 8×10^7 PFU but not until 24 hr after inoculation with 8×10^4 PFU. Two points concerning the effect of the serum interferon are apparent. (i) Once interferon appeared in the circulation, further virus replication in the vascular system was inhibited. (ii) Interferon present in the blood did not prevent subsequent virus replication in

the brain. In both experiments, however, the virus appeared to reach the brain when the serum-virus titer was $\sim 10^5$ PFU/ml. In these experiments this level of viremia occurred either prior to, or concomitantly with, the rise of serum interferon. This may explain why the interferon produced was ineffective in protecting the mice from fatal infection. Since interferon production appears to be associated with extensive viremia, the defensive value of naturally occurring interferon in infections with MM virus may be slight.

The UV irradiation study demonstrated the high sensitivity of MM virus to UV energy and extended previous observations (8) on the sensitivity of interferon-inducing activity to extensive UV exposure. Viable virus was apparently necessary for interferon induction.

The interferon-inducing activity of MM virus in L cells could be increased or decreased by thermal energy (56 C) depending on the length of exposure. Interferon production was dependent on the presence of viable virus after heating. The great increases in interferon-inducing activity of virus heated for 18 hr were unexpected. We cannot at the present time explain this phenomenon. Preliminary experiments minimize the possibility that it was due merely to the presence of a small amount of virus in the inoculum. It was thought that the changes in interferon-inducing activity by heated virus might possibly be due to changing balance between virus and interferon titers seen in Fig. 1 and 2 and virus batches A, B, and C in Table 3. This was also ruled out, however, since virus batch D which contained no detectable interferon also exhibited a large increase in interferon-inducing activity after heat treatment. We are currently investigating the hypothesis that a heat-labile inhibitor of interferon induction might be present in preparations of virus.

Another finding was that a four- to ninefold increase in interferon yield could be achieved by pretreating the cells with homologous interferon. The yield was dependent on the concentration of interferon used for pretreatment, the length of pretreatment, and the multiplicity of infection.

Investigation of the mechanisms of interferon production and its mode of action often require the use of different cell-virus systems to study different aspects. MM virus demonstrates many interferon-inducing characteristics which recommend it for use in interferon studies (i.e., induces relatively high-titered interferon in vitro and in vivo; interferon yields can be increased or decreased by heat, UV irradiation, and pretreatment of cells with homologous interferon; it is sensitive to the action of interferon). A significant factor is that the same virus can be used to study different aspects of the problem.

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