Enhancing Effect of the Murine Sarcoma Virus (MSV) on the Replication of the Mouse Hepatitis Virus (MHV) *in Vitro** (33798)

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The importance of stimulating interactions between viruses has been stressed in several previous reports (1–4). thus, we have shown that all viral functions of Kilham's rat virus (parvovirus ratti) were enhanced when the cells were previously infected by adenovirus 12, SV_{40}, or polyoma virus. This enhancement was related to a protein present in infected or transformed cells which was termed stimulon (stimulating substance).

Previous reports (5–9) indicated a widespread dissemination of MHV in laboratory mouse populations infected with leukemia viruses. These observations suggested the possibility that a similar stimulating interaction might occur between MHV and the leukemia viruses. Results of experiments which demonstrate such a stimulation *in vitro* between MHV and MSV viruses are reported herein (10). The MSV virus was chosen because of its close relationship to the Moloney leukemia virus (11, 12) and because of its marked oncogenicity and the facility of *in vitro* assay.

Materials and Methods. Cells. Primary and secondary mouse embryo fibroblasts from Balb/c mice (MEF Balb) were prepared by the usual technique. A continuous line of mouse "L" cells was routinely maintained in this laboratory. The cells were grown and maintained in Eagle's basal medium with 5 ir 10% heat-inactivated calf serum.

Viruses. West Nile virus obtained through the courtesy of N.B. Finter was employed as a 10% suspension of infected mouse brain. The virus was passaged by intracerebral inoculation of young adult Swiss of IC (Institut de Cancer) mice.

Vesicular stomatitis virus (VSV) Indiana strain was routinely passaged in mouse embryo fibroblasts (MEF). Viral titrations were performed using a standard plaque assay.

Mouse hepatitis virus (MHV_1) strain was obtained through the courtesy of Microbiological Associates; Bethesda, Md. The virus was propagated in newborn Balb/c mice inoculated by intraperitoneal or intramuscular route or in MEF Balb/c cells *in vitro* (13). Infectivity titrations were performed in L cells by plaque assay as follows: Mouse L cells were grown in 5-cm plastic petri dishes. 0.5-ml of tenfold dilution were inoculated in three petri dishes per dilution. After an absorption period of 1 hr the cultures were overlaid with agarose 0.60% in Eagle's basal medium plus 5% calf serum. After 48 hr incubation at 37°, the cells were stained for 5–6 hr with neutral red and the plaques were counted.

Technique for the determination of multiplication of MHV in MEF Balb/c cells: Balb/c fibroblasts grown in 60-ml prescription bottles were inoculated with 1 ml of MHV viral suspension at a multiplicity of infection (m.o.i.) $\cong$ 0.04. After an incubation period of 1 hr, the unadsorbed virus was removed, pooled, and titered. The bottles were placed in a waterproof incubator immersed in an agitated water bath at 37°. At each point 2 bottles were chosen at random and frozen at —80° for titration.

Murine sarcoma virus (MSV) was received through the courtesy of Dr. John Moloney as a tumor extract from infected mice, and virus stocks were prepared by the extraction techniques of Moloney (11). Tumors were obtained by intramuscular inoculation of newborn Balb/c mice. A 10% suspension was made from pooled tumors at 4° with 0.153 *M*

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K-citrate buffer containing 1.5 mg/100 ml of hyaluronidase. After 10 min of initial homogenization the extract was digested for 1 hr with intermittent (1 min) homogenization every 10 min.

After digestion, the material was twice clarified by low speed centrifugation (2400*g* for 20 min and 10,000*g* for 5 min) and subsequently was concentrated by ultracentrifugation at 30,000*g* for 1 hr. The sediment was resuspended at a final concentration of 1 gm equiv/ml in a 0.05 *M* Na-citrate buffer at pH 6.8. The aliquots were rapidly frozen and stored in liquid nitrogen.

Titration of the virus was performed by the *in vivo* (50% tumor induction end point) or the *in vitro* pock forming capacity (14). There was usually a good correlation between the two methods. In the former, newborn Balb/c mice were inoculated intramuscularly in the thigh. Tumors developed at the site of inoculation in 5–10 days ensuing usually in 12–15 days.

Interferon preparation. Preparations of mouse brain interferon were obtained by intracerebral inoculation of IC mice with West Nile virus according to the method of Finter [(as modified by Gresser (15, 16)]. These preparations were concentrated 10-fold by pressure dialysis. Control mouse brain extracts were prepared from uninoculated mice. Interferon was assayed by standard 50% vesicular stomatitis virus plaque reduction techniques using L cells.

Treatment of cells with actinomycin D and puromycin. Cells were treated with actinomycin D (0.01–0.05μg) or puromycin (2μg) for 18 hr. After incubation with either of the inhibitors the cells were pulse labeled for 2 hr with 2μCi/ml of uridine-6-^{3}H or leucine-^{3}H, respectively. The cells were washed and precipitated with trichloracetic acid 5% at 4° for 3 hr. The precipitate was dissolved in NaOH, 0.1 *N*, and incubated at 4° for 24 hr and then counted in a liquid scintillation counter (Packard). The biological action of puromycin was controlled with leucine-^{3}H 2μCi/ml.

Results. Multiplication of mouse hepatitis virus in mouse embryonic fibroblast Balb/c cells. The MHV virus produced plaques only irregularly in MEF Balb/c cells, whereas plaques were constantly observed when L cells were inoculated with the same viral suspension. The multiplication of MHV in MEF Balb/c cells could only be analyzed when the virus produced during the first infectious cycle was titered in L cells.

In Balb/c cells the duration of the eclipse phase was about 8 hr and that of the first infectious cycle about 22 hr. Thus in spite of the irregularity of plaque production (for which several infectious cycles are required) in this system, the yield of infectious virus after the first cycle was of appreciable quantity.

The irregularity of plaque production in MEF Balb/c cells was probably related to the autoinhibitory phenomenon illustrated in Fig. 1. Thus, when two suspensions of MHV

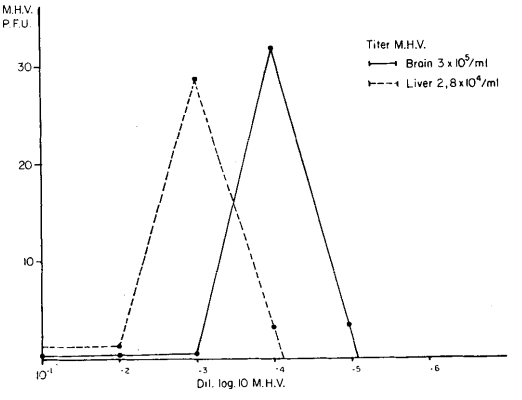

FIG. 1. Titration of 2 different preparations of mouse hepatitis virus (extracted from the brain and liver of infected mice) in mouse embryonic Balb/c cells. A pronounced auto inhibitory zone is observed in both instances.

derived from mouse liver and brain were titered in MEF Balb/c cells as described in "Material and Methods," plaques appeared essentially only at the 10^{-3} or 10^{-4} dilution. When the same brain suspension was titered in L cells such an autoinhibitory zone was not observed.

Stimulation of mouse hepatitis virus multiplication and decrease of the autoinhibition in mouse embryonic fibroblasts previously infected with mouse sarcoma virus. When cultures of mouse fibroblasts were infected with MSV 24 hr before or simultaneously with

MHV, a significant increase in the yield of infectious MHV was observed when compared to control cultures.

Figure 2 shows a typical dose–response curve of MSV expressed as the plaque titer of enhanced MHV.

The enhancing effect of MSV on MHV increased with the concentration of MSV and was optimal at a multiplicity of 0.03 pock forming units/cell. Further augmentation of the MSV concentration abolished stimulating action on MHV. MSV similarly decreased the autoinhibitory zone observed during the titrations of MHV in MEF Balb/cells. Cells were infected with serial dilutions of MSV at multiplicities of 0.3 to 0.00003 pock forming units/cells for 24 hr. One ml of MHV containing 10^2 pfu were then added to each culture and incubated for 48 hr. MHV plaques appeared in cell cultures infected with both viruses at dilutions where no plaques were observed in cells infected with MHV alone (Fig. 3).

FIG. 2. Dose–response curve relating the multiplicity of infection (m.o.i.) of mouse sarcoma virus in pock forming units and the yield of mouse hepatitis virus after 3 cycles of multiplication; 3 × 22 hr.

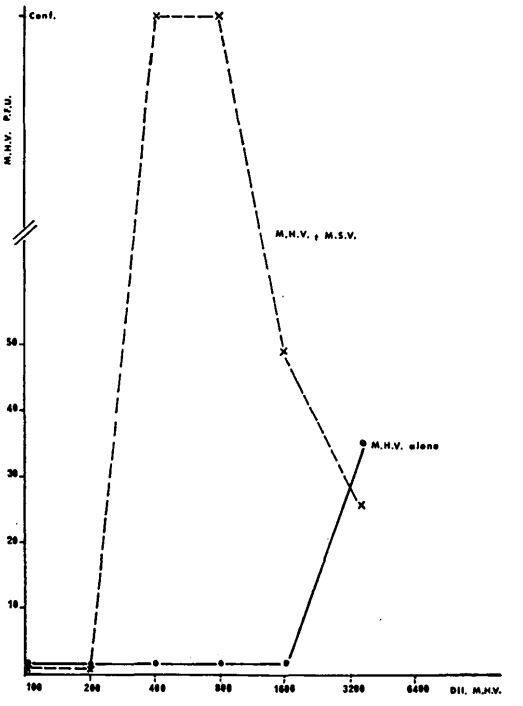

FIG. 3. Decrease of the auto inhibitory zone in mouse embryonic fibroblasts observed in Fig. 1 (with MHV extracted from the brain) when mouse sarcoma virus was inoculated simultaneously with mouse hepatitis virus.

Effect of mouse sarcoma virus on the production of interferone. The decrease of the autoinhibitory effect of MHV by MSV in mouse fibroblasts and the susceptibility of MHV to interferon suggested that the enhancement of MHV by MSV may have been due, at least in part, to an anti-interferon effect induced by MSV. As shown in Table I,

TABLE I. Interferon Production by NDV in MSV Infected Balb/c Mouse Embryonic Fibroblasts.

Group	0 hr	24 hr	Interferon titer[c] Expt. 1	Interferon titer[c] Expt. 2
1	MSV[a]	Medium	<4	<8
2	MSV	NDV[b]	≥256	256– 512
3	Medium	NDV	256	512–1024
4	Medium	MSV	256	256

[a] MSV, multiplicity of infection 0.03.
[b] NDV, multiplicity of infection 100.
[c] Units/ml.

MSV (m.o.i. 0, 03) inoculated 24 hours prior or simultaneously with NDV (m.o.i. 100) in Balb/c mouse embryonic fibroblasts had no detectable effect on the production of interferon.

Effect of mouse sarcoma virus on the action of interferon. Highly potent interferon preparations were tested in MEF Balb/c cells in the presence or absence of MSV virus. When the optimal amount of MSV virus was inoculated 24 hr prior to addition of interferon, the interferon titer was diminished tenfold. When MSV was inoculated 4 or 24 hr after the addition of interferon, the anti-interferon effect still appeared in some experiments (Fig. 4).

Resistance of mouse sarcoma virus to interferon in vivo. The effect of interferon on MSV was also investigated *in vivo* because of the known inhibitory effect of interferon on polyoma virus (17) and on mouse leukemia viruses (15, 16). Table II summarizes the results of 2 experiments on the effect of interferon on MSV oncogenicity in newborn Balb/c mice. Interferon was injected (400–800 inhibitory doses) 4 hr prior to inoculation of MSV (200 tumor inducing units) and subsequently was administrated daily (1600 inhibitory doses) for 6–10 days. Control mice were treated simultaneously with normal brain extracts. In these experiments interferon treatment did not diminish either the size of the tumor, the time of tumor appearance, nor did it affect the number of mice developing tumors.

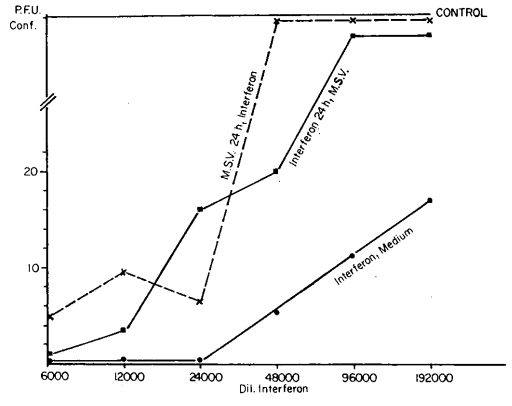

FIG. 4. Anti-interferon effect of mouse sarcoma virus inoculated 24 hr prior to or 24 hr after addition of interferon.

Effect of ultraviolet irradiation and heat treatment on the stimulating and anti-interferon effects of mouse sarcoma virus. MSV was irradiated with a UV light at a total dose of 2000, 5000, or 10,000 ergs/mm^2. Irradiation at 2000 and 5000 ergs/^{2} did not affect the oncogenicity. After irratiation with 10,000 ergs/mm^2 a slight diminution in tumor incidence and a delay in tumor appearance was observed when compared to control mice (Table III).

The anti-interferon capacity of the virus however was completely abolished by irradiation with 2000 ergs/mm^2. Heating of the crude MSV preparation at 56° for 1 hr abolished both the anti-interferon effect and the oncogenicity of MSV (Table III).

Effect of actinomycin D and puromycin on

TABLE II. Effect of Interferon Treatment on the Production of Tumors in Mice Infected with Mouse Sarcoma Virus.

Expt. no.	Length of treatment (days)	Interferon doses	Results: no. of tumors/no. of animals
1	6	1 local injection (400 doses) 4 hr before the virus and subsequently 1 injection ip /24 hr (400 doses)	10/10
		Normal brain extract	16/19
2	10	1 local injection (800 doses) 4 hr before the virus and subsequently 1 injection ip/24 hr (1600 doses)	23/23
		Normal brain extract	23/23

TABLE III. Effect of UV Irradiation and Heating of MSV on Its Anti-interferon and Tumorigenic Function.

MEF Balb/c cells	VSV (no. of plaques) Mean[a]	Range	Tumors (newborn Balb/c mice)	Incubation (days)	Death (days)	No. of tumors /no. of animals
1 Control	42	31–47				
2 MSV	C[b]	C–C	MSV	15	20	13/13
3 Interferon	12	10–15	ND[c]			ND
4 Interferon + MSV	C	C–C	ND			ND
5 Interferon + MSV 2000 erg	11	8–15	MSV 2000 erg	16	18	13/13
6 Interferon + MSV 5000 erg	11	7–17	MSV 5000 erg	16	18	9/9
7 Interferon + MSV 10,000 erg	13	6–28	MSV 10,000 erg	37	50	9/13
8 Interferon + MSV 56°	9	4–21	MSV 56°			0/6

[a] Mean value of 6 petri dishes.
[b] Confluent.
[c] Not done.

TABLE IV. Effect of Actinomycin D on the Enhancement of Mouse Hepatitis Virus by Mouse Sarcoma Virus in Balb/c Mouse Embryonic Fibroblasts.

	MHV	MHV + MSV	% Inhibition cellular protein synthesis
Actinomycin D, 0.01 μg	4.2×10^6	1.7×10^7	50
Actinomycin D, 0.05 μg	6.2×10^5	2×10^6	96
Control	10^7	7×10^7	0

the stimulating function of mouse sarcoma virus. Cells were treated simultaneously with MSV and actinomycin D (0.01 or 0.05 μg) for 18 hr. The effect of actinomycin D on cells metabolism was evaluated with uridine-6-^{3}H as described in material and methods. Table IV shows that after actinomycin D treatment a 2–4-fold enhancement of MHV was observed (despite reduction in protein synthesis of 50 and 96%, respectively) compared to a 7-fold enhancement in control cultures.

When under similar conditions puromycin (2 μg) was employed the enhancing capacity of MSV was completely blocked (Table V). This amount of puromycin blocked 57% of the incorporation of leucine-^{3}H.

Discussion. The frequent spread of MHV in mice infected with leukemia viruses suggested a possible stimulating interaction between mouse leukemia viruses and MHV. The *in vitro* experiments presented above demonstrated that the Moloney strain of MSV enhances the replication of MHV *in vitro*. This stimulating effect was probably induced by the MSV genome. The following observations support this interpretation: (i) Extracts from normal uninfected muscle had no enhancing effect. (ii) Mild UV treatment of MSV easily dissociated the enhancing and tumorgenic capacities of the virus. Thus the stimulating effect of MSV was far more sensi-

TABLE V. Effect of Puromycin on the Enhancement of Mouse Hepatitis Virus by Mouse Sarcoma Virus in Mouse Embryonic Fibroblasts.

	MHV (10^2 pfu)[a]	
	Expt. 1	Expt. 2
MSV (1.4×10^4)[b]	1.3×10^7	1.2×10^7
MSV (1.4×10^4) puromycin (2 μg)	4.6×10^6	1.8×10^6
Medium	6×10^6	8×10^5
Puromycin (2 μg)	6×10^6	1.6×10^6

[a] Plaque forming units.
[b] Pock forming units.

tive to UV irradiation than the moiety of the genome responsible for oncogenicity. (iii) Incubation of the cells with MSV and actinomycin D had little effect on the enhancing capacity of the virus. (iv) Puromycin blocked its stimulating function.

In summary if the integrity of cellular DNA was not required for the stimulating effect of MSV, the integrity of the viral genome and the functional integrity of cellular ribosomes were essential.

This enhancing effect of MSV and MHV could be attributed to two different mechanisms: (i) MSV might facilitate an early step during the infectious cycle of MHV, such as penetration or decapsidation. Such a step would not be inhibited by actinomycin (18). (ii) MSV blocks the action of interferon.

The data reported herein supports the latter hypothesis since MSV inhibited the action of potent interferon preparations, and had no effect on the production of interferon by NDV. This anti-interferon effect was similar to that observed in previous experiments with adenoviruses (1–4). In these experiments it was shown that viral enhancement was mediated by an anti-interferon substance (stimulon). To date observations on the enhancing effect of one virus on the multiplication of a second virus have been confined to DNA viruses. It is therefore of interest to note that this phenomenon can now be extended to RNA viruses. In addition as shown by Peries (19) cells chronically infected with Rauscher and Moloney viruses become resistant to the action of interferon. Consequently it seems to us worth emphasizing that the stimulating interaction between viruses may prove of value in the detection of latent viruses, in a manner similar to that observed for inhibiting interactions (i.e., rubella).

Summary. The Moloney strain of mouse sarcoma virus (MSV) enhances the replication of mouse hepatitis virus (MHV) in Balb/c mouse embryonic fibroblasts (MEF Balb/c).This enhancing effect may explain in part the unusual spread of MHV in leukemic mice, as often reported in the literature. This stimulating effect was induced by the MSV genome and seems to be related to a new protein produced by the cells. The data here presented support the hypothesis that this stimulating activity was related to a blocking effect of this protein on the biological action of interferon, comparable to the stimulons previously described for adenoviruses. *In vivo* MSV appeared to be resistant to highly potent interferon preparations, *in vitro* it diminished the titer of exogenous interferon. MSV had no detectable effect on the production of interferon in MEF Balb/c cells by Newcastle disease virus (NDV).

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