Amantadine Hydrochloride: Inhibitory Effect on Murine Sarcoma Virus Infection in Cell Cultures[1] (36342)

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Amantadine hydrochloride (1-adamantanamine hydrochloride) has been shown to inhibit certain myxoviruses and several strains of human influenza and parainfluenza viruses *in vitro* and *in vivo* (1–4). The evaluation of amantadine in the prevention and treatment of influenza A_2 in humans has been widely discussed recently (5). Other viruses such as rubella and pseudorabies are also sensitive in one or more systems (6–9). Wallbank *et al.* (10) have found that amantadine has inhibitory effect on Rous sarcoma virus (RSV) infection in cell cultures.

The development of *in vitro* (tissue culture) assay systems for murine leukemia (11) and sarcoma (MSV) viruses (12–14), the demonstration of the defectiveness of the MSV genome (12), and its rescue with various murine leukemia viruses (15), have brought out certain remarkable similarities between these viruses and those of the avian leukosis-RSV complex (12, 15, 16). Therefore it appeared pertinent to determine the action of amantadine on murine sarcoma viruses. The results of our study indicate that amantadine has significant inhibitory effect of MSV infection in cell cultures.

Materials and Methods. Virus. Concentrates of mouse tumors (17) induced by Moloney sarcoma (M-MSV), M-MSV(ML), and M-MSV(FL) pseudotype virus (15) were used in this work.

Cell culture and media. Primary cultures of Swiss NIH mouse embryo tissue culture (NIH-METC) were prepared as previously described (11). Growth and maintenance medium was 10% fetal bovine serum in Eagle's minimum essential medium with 2 m*M* glutamine, and 100 units of penicillin and 100 μg of streptomycin/ml.

Virus assay. Murine sarcoma viruses were assayed in secondary NIH-METC plate cultures. Altered cell foci (12) were counted with an inverted Zeiss microscope 6–9 days after sarcoma virus inoculation. Murine leukemia virus was also assayed in secondary NIH-METC plate cultures by the complement fixation test for murine leukemia viruses, or COMuL test (11, 16).

Complement-fixation. Complement-fixation (CF) tests were carried out by the microtiter technique described for tumor antigen studies (18). Titers were recorded as the reciprocal of the highest dilution giving 3+ to 4+ fixation of 1.8 units of complement. Rat antisera used in the CF test were obtained from Fisher rats carrying transplanted sarcomas induced by M-MSV.

Amantadine preparation. The amantadine hydrochloride (*Symmetrel*), used in this work, was generously provided through the courtesy of E. I. Dupont Co., Wilmington, DE. Stock solution was prepared as 1 mg/ml of amantadine diluted in growth medium. For the experiments, amantadine was further diluted into different concentrations, using growth medium as diluent.

Results and Discussion. Secondary NIH-METC cell suspensions were planted in media containing various concentrations of amantadine. After 10 min of incubation at 37°, the cultures were inoculated with sarcoma viruses. The next day the cultures were washed twice with Hanks' balanced salt solution and supplied with the same concentrations of amantadine received at planting.

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TABLE I. Inhibition of Focus Formation and Complement-Fixing (CF) Antigen Induced by Mouse Sarcoma Viruses in NIH Mouse Embryo Cells in the Presence of Amantadine.[a]

Virus	Virus dilution	Amantadine (μg/ml)	Foci/plate (av. of 2 plates)	CF titer[b]
M-MSV (ML)	$\frac{1}{2} \times 10^{-1}$	100	0	<4
		50	0	<4
		25	5	1:4
		0	208	>8
M-MSV (FL)	$\frac{1}{2} \times 10^{-1}$	100	0	<4
		50	0	<4
		25	35	1:4
		0	280	>8
M-MSV	10^{-1}	100	0	—[c]
		50	1	—
		25	7	—
		0	158	—

[a] Cells were planted in nutrient medium containing various amounts of amantadine and were incubated for 10 minutes at 37°. Virus was inoculated immediately. The next day, cultures were washed and supplied with the amantadine medium.

[b] CF titer = reciprocal of dilution giving 3+ to 4+ fixation of 1.8 units of complement.

[c] — = not done.

They were then incubated for 9 days at 37° in a humidified air atmosphere containing 5% CO_2. The medium was changed every 2 days. Foci of transformed cells were counted at 6 to 9 days. On day 9 after inoculation, the infected cultures were harvested. The fluids were assayed for infectivity, and the cell pack (10×) preparations for the CF test were made by the method described previously (19). Table I shows the focus-forming and CF titers of M-MSV, M-MSV(MLV), and M-MSV(FLV) in METC with and without amantadine medium. Significant inhibitory effects on the viral transforming activity were produced by amantadine. Inhibition of the CF antigen activity was also demonstrated in the MSV infected cultures. The effect was maximal with more than 50 μg of amantadine/ml, though 25 μg also gave a significant effect (Table I). This viral inhibitory effect was further evidenced by the yields in experiments with sarcoma virus-infected METC. In the NIH-METC, in the presence of a 25 μg/ml dose of amantadine, the yield of sarcoma viruses was also reduced by at least 2 logs (Fig. 1). There was no evidence of toxic effect on the cells with the 50 μg/ml dose during the experiment period. However, concentrations greater than 100 μg/ml of amantadine medium did produce a toxic effect. Cultures with 50 μg/ml or, more often, with 100 μg/ml exhibited slight morphological changes as the treatment period lengthened. Larger cells of more granular nature appeared, along with slowness of growth, and it

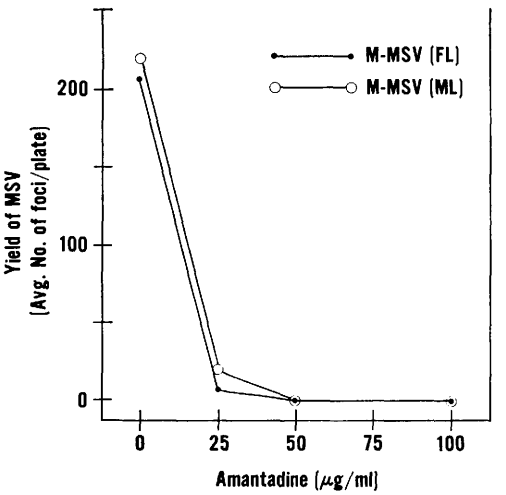

FIG. 1. Yield of foci produced by mouse sarcoma viruses in mouse embryo cells after 9 days incubation with and without amantadine medium: The multiplicity of input was 0.01.

TABLE II. Effect of Amantadine on Suspensions of M-MSV at 37° for 2 hr.

Amantadine HCl[a] (μg/ml)	Focus-forming units (per 0.2 ml; × 10^3)
100	2.4
50	4.9
25	6.5
0	6.4
0[b]	6.5

[a] Viral suspensions were mixed with various concentrations of amantadine or nutrient medium. The mixture was held at 37° for 2 hr, then used to infect monolayers of NIH-METC in order to determine focus-forming units.

[b] Before incubation at 37° for 2 hr.

was difficult to count the foci in these cultures. This difference from the usual appearance of foci suggest that mouse embryo cells were affected in some way by the drug. The same morphological changes were observed with this compound (50 μg/ml) in chick cells by Wallbank *et al.* (10).

The possibility of virucidal effect of amantadine was examined. Viral suspensions were mixed with various concentrations of amantadine or nutrient medium. The mixture was held at 37° for 2 hr, then used to infect monolayers of NIH-METC in order to determine focus-forming units. The results are shown in Table II. No measurable or significant virucidal effect on MSV in suspension occurred with the amantadine at the concentrations used in these experiments, except 100 μg/ml of amantadine (0.4 log reduction of virus). There is evidence to show that amantadine acts by blocking the penetration of virus into the cells (9). However, there is also good evidence to show that the antiviral action of amantadine was due to an inhibition of uncoating of virus in the cells (20).

Our experiments clearly demonstate the effectiveness of amantadine in the inhibition of MSV infection in cell cultures. Optimal conditions for inhibitory effect of amantadine on MSV infection *in vitro* are under investigation. However, the preliminary results of *in vivo* experiment with amantadine indicated that the inhibitory effect on MSV in mice was equivocal.

Summary. Amantadine hydrochloride (1-adamantanamine hydrochloride), a compound reported to be active against influenza viruses and Rous sarcoma viruses, inhibited the *in vitro* cellular transformation induced by Murine sarcoma viruses. The antiviral activity is not due to direct inactivation of the virus.

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