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RIMANTADINE BUT NOT AMANTADINE PROTECTS FISCHER RAT EMBRYO CELLS FROM TRANSFORMATION INDUCED BY 3-METHYLCHOLANTHRENE OR BENZO(a)PYRENE

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SUMMARY

The antiviral drugs amantadine hydrochloride and rimantadine hydrochloride were tested as to their oncogenic potential using a serial line of Fischer rat embryo cells that previously had been shown to be an accurate indicator of chemicals known to be oncogenic in animal studies. Neither compound was found to have transforming activity. At slightly toxic levels, rimantadine hydrochloride, but not amantadine hydrochloride, protected the same cell line from the transformation induced by the polycyclic hydrocarbons 3-methylcholanthrene and benzo(a)pyrene.

Key words: transformation; antiviral drugs; amantadine hydrochloride; rimantadine hydrochloride.

INTRODUCTION

Amantadine hydrochloride (tricyclo-[3,3,1,1]-decan-1-amine hydrochloride) and its analogue rimantadine hydrochloride (α-methyl-1-adamantanemethlamine hydrochloride) are drugs reported to be active against influenza viruses both in vivo and in vitro (1). It has been suggested that their antiviral activity is the result of their ability to prevent viral uncoating (2). Amantadine hydrochloride (Am) also has been shown to be ineffective in inhibiting Gross murine leukemia virus replication in Swiss mouse embryo cells (3), but effective against Rous sarcoma virus (RSV) in chick embryo fibroblasts (4). Rimantadine hydrochloride (Rm) also has been shown to be effective in reducing focus formation of RSV in chick embryo cells (5).

Since Am has been approved for the systematic management of influenza A infection in the USA, and Rm for the same use in the USSR (Dr. C. E. Hoffman, *personal communication*), we were interested in examining their in vitro oncogenic potential and/or ability to protect cells from transformation induced by polycyclic hydrocarbons. We report here that neither compound is a transforming agent for a line of Fischer rat embryo cells that previously had been shown to be an accurate and sensitive indicator of chemicals having oncogenic properties (6). Further, at slightly toxic levels, Rm, but not Am, protects the same cell line from transformation induced by the known polycyclic hydrocarbon carcinogens 3-methylcholanthrene (MCA) and benzo(a)pyrene (B(a)P).

MATERIALS AND METHODS

Toxicity testing. Reduction in plating efficiency relative to a medium control was used to determine the toxicity of Am and Rm. Eagle's minimum essential medium in Earle's salts, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U per ml penicillin and 100 μg per ml streptomycin, was used as the control medium. In order to determine the plating efficiency, 60-mm plastic cell-culture dishes (Lux) in triplicate each were inoculated with 500 cells of the Fischer rat embryo cell line F1706 at the 95th population doubling (D95). The dishes were incubated overnight at 37° C in a humidified 5% CO_2-in-air incubator. The next morning the medium was decanted and replaced with either control medium or control medium now containing serial dilutions of each chemical which initially had been diluted to 400 μg per ml in sterile deionized water, filtered

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through a 0.45-μm nalgene filter, and then diluted directly into the medium. Five days later the dishes were fixed and stained (methylene blue-carbol fuchsin), and macroscopic colonies were counted.

Transformation assay. Transforming potential was determined in two separate experiments, each run in duplicate. F1706 cells (D97 in the first experiment and D95 in the second) were inoculated onto plastic flasks at a concentration of 1000 cells per ml. The next day the cultures were refed with growth medium alone or with growth medium that had been supplemented with either 0.5 μg per ml Rm (both experiments), 1.0 μg per ml Am (second experiment) or 2.0 μg per ml Am (first experiment). Three days later the cells from each group were transferred to new flasks in a medium containing the drug at the given concentration. In the first experiment the cells were plated at a concentration of 20,000 cells per ml and in the second at 1000 cells per ml. The next morning the cultures were refed with their respective medium, and the ability of each drug to induce transformation, or to protect against transformation, was determined by adding 1 ml of a 15X concentration of MCA (final concentration of 0.2 μg per ml in the first experiment and 0.1 μg per ml in the second) or B(a)P (final concentration of 0.5 μg per ml). Three days after treatment with the known carcinogens, the cultures were washed and refed without carcinogen, Rm or Am. The next day new cultures were initiated at 1000 cells per ml in complete growth medium.

This treatment schedule resulted in the following sets of cultures: medium only (negative control); 0.1 or 0.2 μg per ml MCA and 0.5 μg per ml B(a)P (positive controls); 0.5 μg per ml Rm; 1.0 μg per ml or 2.0 μg per ml Am; Rm plus MCA or B(a)P; and Am plus MCA or B(a)P. At each subculture following the initial treatment, one set of flasks was set aside to be held without subdivision (holding series), and the other set subdivided 1:2 weekly to provide two new sets of cultures (one for the holding series and one for subdivision). Both the holding and subdivided series received fresh growth medium twice a week. Transformation was determined by the appearance of foci of cells lacking contact inhibition and orientation, and by the formation of macroscopic colonies in semisolid agar.

RESULTS

It has been our experience that the optimal transforming dose in the Fischer rat embryo cell transformation assay system (F1706) is approximately the LD10-30 (level reducing the RPE or relative plating efficiency by 10% to 30%). As can be seen in Table 1, 2.0 μg per ml amantadine hydrochloride reduced the RPE by 28% (level used in the first experiment), whereas 1.0 μg per ml reduced the RPE by 12% (level used in the second experiment). Rimantadine hydrochloride was more toxic; 1.0 and 2.0 μg per ml Rm reduced the RPE by 33% and 42%, respectively. In both experiments, a level of 0.5 μg per ml of Rm was selected (level reducing the RPE by 12%).

TABLE 1

TOXICITY TESTING OF RIMANTADINE (RM) AND AMANTADINE (AM) AS DETERMINED BY REDUCTION OF MACROSCOPIC COLONY FORMATION OF FISCHER RAT EMBRYO CELLS (F1706 D95)[a]

	Concentration	Colonies per Dish[b]	Relative Plating Efficiency[c]
	μg/ml		%
Rm	20	0	0
	10	4	4
	5	11	10
	2	63	58
	1	72	67
	0.5	95	88
Am	20	0	0
	10	6	6
	5	26	24
	2	78	72
	1	95	88
	0.5	101	94
Control medium		108	100

[a] D = population doublings.
[b] Average number of macroscopic colonies per three dishes. Each dish was inoculated with 500 cells.
[c] Percentage of cells giving rise to macroscopic colonies relative to the control medium in which the plating efficiency was arbitrarily set at 100%.

In both experiments, each run in duplicate, neither Am at either level nor Rm transformed the cells (Table 2). Cultures treated with either drug were still phenotypically normal at the end of the experiments, 9 or 10 subcultures after treatment (D+9 or D+10). Cells inoculated into semisolid agar did not form macroscopic colonies when tested at D+3, D+8 or D+10 in the first experiment, or at D+3, D+6 and D+9 in the second. However, as expected, cells treated with the known carcinogens MCA or B(a)P were transformed. In the first experiment, cells treated with 0.2 μg per ml MCA were phenotypically transformed by the second vertical subculture (D+2) in one series and at the sixth D+6) in the other,

TABLE 2

3-METHYLCHOLANTHRENE (MCA) AND BENZO-a-PYRENE (B(a)P)-INDUCED TRANSFORMATION OF F1706 FISCHER RAT EMBRYO CELLS AND PROTECTION FROM TRANSFORMATION BY RIMANTADINE (RM) BUT NOT AMANTADINE (AM)

Treatment (per ml)	Experiment I				Experiment II			
	Morphological transformation		Growth in agar[a]		Morphological transformation		Growth in agar[a]	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Medium control	−(+10)	−(+10)	−(+10)	−(+10)	−(+9)	−(+9)	−(+9)	−(+9)
0.5 μg/ml Rm	−(+10)	−(+10)	−(+10)	−(+10)	−(+9)	−(+9)	−(+9)	−(+9)
1.0 μg/ml Am	ND[b]	ND	ND	ND	−(+9)	−(+9)	−(+9)	−(+9)
2.0 μg/ml Am	−(+10)	−(+10)	−(+10)	−(+10)	ND	ND	ND	ND
0.1 μg/ml MCA	ND	ND	ND	ND	+(+3)	+(+7)	+(+3)	+(+6)
0.2 μg/ml MCA	+(+2)	+(+6)	+(+10)	+(+10)	ND	ND	ND	ND
0.5 μg/ml B(a)P	ND	ND	ND	ND	+(+3)	+(+3)	+(+3)	+(+3)
0.5 μg/ml Rm + 0.2 μg/ml MCA	−(+10)	−(+10)	−(+10)	−(+10)	ND	ND	ND	ND
2.0 μg/ml Am + 0.2 μg/ml MCA	+(+8)	+(+8)	+(+8)	+(+8)	ND	ND	ND	ND
1.0 μg/ml Am + 0.1 μg/ml MCA	ND	ND	ND	ND	+(+8)	+(+9)	+(+9)	−(+9)
1.0 μg/ml Am + 0.5 μg/ml B(a)P	ND	ND	ND	ND	+(+4)	+(+5)	+(+9)	+(+9)
0.5 μg/ml Rm + 0.1 μg/ml MCA	ND	ND	ND	ND	−(+9)	−(+9)	−(+9)	−(+9)
0.5 μg/ml Rm + 0.5 μg/ml B(a)P	ND	ND	ND	ND	−(+9)	−(+9)	−(+9)	−(+9)

[a] Triplicate agar dishes were each inoculated with 50,000 cells from cultures at D + 3, D + 8 and D + 10 in the first experiment, and at D + 3, D + 6 and D + 9 in the second experiment. (D = population doublings after removal of the chemical.) The cultures were held 4 weeks at 37° C in a humidified 5% CO_2 incubator, and then screened for the presence of macroscopic colonies.

[b] Not done.

and formed macroscopic colonies in semisolid agar (D+10). In the second experiment, the cells were treated with either MCA at a level of 0.1 μg per ml or B(a)P at a level of 0.5 μg per ml. Cells phenotypically altered by MCA were first noted in one series (*A*) at D+3, and in the second series (*B*) at D+7. MCA-treated cells from the *A* series formed macroscopic colonies in semisolid agar when tested at D+3, D+6 and D+9, whereas those from the *B* series were negative at D+3 but positive at D+6 and D+9. Both series of cells treated with B(a)P were phenotypically altered at D+3, and formed macroscopic colonies in semisolid agar at D+3, D+6 and D+9. All positive dishes had a minimum of 12 colonies per dish.

When the cells were treated with Am prior to and during treatment with each of the carcinogens (MCA or B(a)P), the expression of transformation was delayed, but transformation was not eliminated. Cells dually treated with Am and MCA were transformed by the eighth or ninth subcultures, and with Am and B(a)P by the fourth or fifth subculture. In contrast, cells treated with Rm prior to and during treatment with MCA or B(a)P remained phenotypically normal and did not grow in semisolid agar (Table 2).

DISCUSSION

We have previously reported on the use of the Fischer rat embryo F1706-cell-transformation assay system as both a prescreen for potentially oncogenic chemicals (6) and for chemicals having anticancer properties (8-11). All compounds that we examined, which had the ability to protect the cells from carcinogen-induced cell transformation, also had anti-oncornavirus properties. Streptonigrin (8), cordycepin (9) and geldanamycin (10) protected the cells from chemically induced transformation, and also inhibited the induction of measurable endogenous rat leukemia virus by halogenated pyrimidines. 9-B-D-Arabinofuranosyladenine (Ara-A) did not inhibit the induction of the endogenous xenotropic virus, but previously had been shown to be an efficient inhibitor of the replication of ecotropic oncorna viruses (3).

One possible hypothesis is that the oncorna virus is turned on by the chemical carcinogen and

then induces transformation by acting as a means of transferring and modulating cellular oncogene sequences. If this is the case, then the protective action of Rm and the other antiviral antibiotics studied may be due to the inhibition of either chemically induced oncorna-viral turn-on, spread, absorption, penetration, uncoating or integration. The accumulated data neither prove nor disprove such a hypothesis.

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