STUDIES ON THE ANTIVIRAL ACTIVITY OF AMANTADINE HYDROCHLORIDE*

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Amantadine, or 1-adamantanamine hydrochloride, has been reported to possess activity against influenza virus *in vitro*, in mice and in man,[1-4] and against rubella virus *in vitro*.[5] The scope and mechanism of its activity, therefore, are of particular interest. This report describes the activity of amantadine against other respiratory viruses and offers new information concerning its site of action.

Materials and Methods

Tissue culture techniques. HeLa cells were infected with strains of parainfluenza viruses, types 2 (Greer) or 3 (C 243), obtained from R. M. Chanock, National Institutes of Health, Bethesda, Md. The LLC-MK_2 line of monkey kidney cells[6] was used for parainfluenza 3 and the WM strain of rubella viruses.[7] Other studies with rubella virus involved the chronically infected line previously described.[8] The Long strain of respiratory syncytial virus, obtained from the American Type Culture Collection and grown in the H-L line of human epithelial cells, and the Japan 305 strain of influenza virus grown in primary calf kidney cells, were also used. Virus was detected by hemagglutination of guinea pig erythrocytes, by titration of infectivity, or by cytopathogenic effect, depending on the particular cell-virus system. Antiviral activity was evaluated either by comparison of virus yield from treated and control cultures when measured by relative cytopathology, hemagglutination, or infectivity titrations, or by calculation of a therapeutic index.[9]

In vivo *techniques.* Swiss Webster mice or yearling ferrets were infected with influenza virus by a 30-minute exposure to aerosols of diluted suspensions of infected mouse lungs. The WR strain of vaccinia virus was given to mice intranasally. Disease was evaluated either by lung lesion scores[10] or by mean survival times.[1] X-ray was given as previously described.[11]

Results

Inhibition of myxoviruses in vitro. The problems of diagnosis and estimation of effectiveness make it important to assess the susceptibilities of all pneumotropic infectious agents to a drug proposed for any respiratory disease. In addition, the sharing of susceptibility to amantadine by several influenza viruses[1] and rubella virus,[5] together with the noted similarities[12] of rubella virus to the myxovirus group, prompted exploration of inhibition by amantadine as a property common to myxoviruses. Accordingly, the antiviral activity of amantadine for several additional myxoviruses was studied, with the results presented in TABLE 1. The HeLa, LLC-MK_2 and HL cell lines maintained in our laboratory all tolerated 62.5 μg. of amantadine hydrochloride per ml., a concentration not directly virucidal to any of the viruses used in these experiments. Amantadine hydro-

* These studies were aided by grants from The National Foundation; the National Institute of Allergy and Infectious Diseases (AI 05876-01); the Office of the Surgeon General, U. S. Army, under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board; and by an Eli Lilly fellowship awarded to Dr. Tsunoda.

TABLE 1

INHIBITION OF MYXOVIRUS *in vitro* BY AMANTADINE HCl

Cell-virus system	Day after virus	Hemagglutinin, 1/n			Infectivity, 10-n		
		Amantadine HCl, μg./ml.			Amantadine HCl, μg./ml.		
		0	51.2	62.5	0	31.2	62.5
Parainfluenza-2 vs. HeLa, 10:1	0	0	0	0	2.7	3.0	3.0
	2	4	4	4	5.0	4.5	3.3
	4	16	16	16	7.0	6.5	5.3
	(CPE)				(4+	3+	1+)
Parainfluenza-3 vs. HeLa, 100:1	1	0	0	0	5.7	5.0	4.5
	2	16	16	16	6.5	6.0	4.7
	(CPE)				(4+	4+	4+)
Parainfluenza-3 vs. HeLa, 10:1	3	16	12	4	6.7	6.3	5.0
	(CPE)				(4+	3+	1+)
Parainfluenza-3 vs. LLC-MK_2, 10:1	0	0	0	0	2.7	2.5	2.5
	2	8	2	0			
	4	64	32	8	5.3	4.7	3.0
	6	64	64	16	6.5	6.0	4.7
Resp. synct. vs. HL, 10:1	0				3.0	3.2	3.4
	2				6.8	6.5	4.8
	(CPE)				(4+	3+	1+)

chloride in concentrations up to 62.5 μg./ml. did not inhibit hemagglutinin production in HeLa cells inoculated with parainfluenza-2 virus at a multiplicity of 10:1. However, when virus yield was measured by infectivity or cytopathology, there was inhibition, especially at the higher concentration. With parainfluenza 3 virus at a multiplicity of 100:1, measurements of hemagglutinin or cytopathic activity did not disclose the inhibition seen in the production of infectious virus. At a lower multiplicity of parainfluenza 3 virus of 10:1, all three measurements—hemagglutination, infectivity, and cytopathology—revealed inhibition. With parainfluenza 3 virus in LLC-MK_2 cells at a multiplicity of 10:1, inhibition of virus production was observable whether measured by infectivity or hemagglutination. The ability of amantadine hydrochloride to inhibit respiratory syncytial virus was detectable by measurement of either the yield of infectivity or by cytopathology. These studies show that amantadine hydrochloride can inhibit selected myxoviruses other than influenza when used at high enough concentrations in a suitable host-virus system and with sensitive methods of following virus.

Activity in mice and ferrets. Other experiments were directed toward exploring the activity of amantadine *in vivo*. Data showing the protective effect of amantadine hydrochloride against aerosol-induced influenza are presented in TABLE 2. It is noteworthy that the Lee strain of type B influenza was also susceptible. Vaccinia virus, included because of a recent report of its susceptibility in plaque inhibition tests,[13] was given by intranasal injection. Vaccinial lung lesions were not found to be inhibited.

The effectiveness of amantadine *in vivo* had been demonstrated only in mice[1] before its trial in man.[2] Since more than one laboratory animal is usually desirable, amantadine was also tested for activity against influenza using ferrets. Amantadine hydrochloride is relatively more toxic for ferrets than for mice. Whereas 233 mg./kg. given intravenously is an LD_{50} for mice,[1] all ferrets receiv-

TABLE 2

ANTIVIRAL ACTIVITY OF AMANTADINE HCl IN MICE

Virus	Average lung lesion score*		Per cent decrease
	Treated†	Control	
Influenza, PR 8	1.6	2.6	38
Influenza, A_2/AA/2/60	.5	1.2	58
Influenza, B/Lee	2.7	3.4	21
Vaccinia, WR	2.4	2.5	4

* Scored 5 for spontaneous death, and 1 for each 25 per cent of lung consolidation[10] on the fifth day after the virus, (seventh day for B/Lee).

† 100 mg./kg./day of amantadine HCl, given subcutaneously for four days starting one day after virus.

ing a single intraperitoneal dose of 200 mg./kg. of amantadine hydrochloride suffered convulsions and died in approximately 30 minutes. Daily intraperitoneal doses of 100 mg./kg. were tolerated for only two days, but given orally in two daily doses, the same amount was tolerated by ferrets for at least 13 days. Results of treating ferrets infected by aerosols of PR8 influenza virus are given in TABLE 3. From these results it may be seen that in ferrets amantadine hydrochloride aggravated rather than alleviated infection with influenza virus.

Mechanism of action of amantadine: Influence of host immunity. Davies *et al.*[1] showed the combination of amantadine with specific antiserum to be more effective against influenza *in vitro* than either treatment separately. This finding and other impressions[14] suggested that antibody might also participate in the antiviral activity of amantadine during the early stages of the *in vivo* infection. To test this hypothesis amantadine was studied in mice whose capacity for antibody production was impaired by a large sublethal dose of X-ray. Results of two experiments, given in TABLE 4, show that a single whole-body exposure to 350 R one day before virus, or treatment with amantadine hydrochloride starting two days before virus, provided protection as indicated by the increases in mean survival time. Results obtained with combined amantadine and X-ray treatment indicated

TABLE 3

ENHANCEMENT OF INFLUENZA IN FERRETS BY AMANTADINE HYDROCHLORIDE

Treatment*	Treated		Control	
	Mortality	Lung damage†	Mortality	Lung damage
50, SQ × 13	2/3	Fd 6 ++++	0/3	+++
		Fd 9 ++++		+
		±		+++
50, SQ × 12	1/3	Fd 7 ++++	0/3	+
		++++		+
		+++		++
100, Oral × 6	3/3	Fd 6 ++++	0/3	±
		Fd 7 ++++	0/3	0
		Fd 7 +++		0

* Mg./kg./day given subcutaneously (SQ) once daily or orally (in two equal doses) starting the day after virus exposure. Control animals received equal volumes of buffered saline.

† Lung damage scored as in TABLE 2.

TABLE 4

SPARING EFFECT OF X-RAY AND AMANTADINE HCl ON PR 8 INFLUENZA IN MICE

Treatment	Mean survival* (days)
None	6.2, 6.6
X-ray†	7.5, 8.2
Amantadine HCl‡	8.7, 8.4
Amantadine HCl + x-ray	9.3, 8.8

* Number of mice alive each day to day 17.[1]
Total number of mice in group

† 350 R, given in a single dose, one day before virus.

‡ 100 or 50 mg./kg./day, subcutaneously × 20, starting two days before virus.

that their individual effects were additive. Other data from appropriate control groups, not given in the Table, showed that amantadine had no significant influence on the radiosensitivity of mice.

Locus of antiviral activity. On the basis of its time of effectiveness and comparison with specific antiserum, it was concluded[1] that the site of action of amantadine was the penetration by the virus of the host cell. The possibility that amantadine might be antagonizing an amino acid led to attempts to reverse its effect with several amino acids. Proline, tyrosine, phenylalanine, or tryptophane failed to alter the ability of amantadine to inhibit influenza virus cytopathology in primary calf kidney cell cultures.

Additional information was sought through comparison of the inhibitory activities of amantadine hydrochloride and aminophenylmethane sulfonic acid, the latter a type of inhibitor of influenza virus[15] whose locus of action has been established to be early in the infectious cycle at adsorption or penetration.[16] The results, presented in TABLE 5, showed that in primary calf kidney cells infected with Japan 305 influenza virus, either amantadine hydrochloride or aminophenylmethane sulfonic acid was ineffective applied postinfection; that either was equally

TABLE 5

CROSS TREATMENT OF INFLUENZA VIRUS (A_2/JAPAN 305) WITH AMANTADINE HYDROCHLORIDE AND AMINOPHENYLMETHANE SULFONIC ACID IN PRIMARY CALF KIDNEY CELL CULTURES

Treatment*		
Amantadine hydrochloride (62.5 γ/ml.)	Aminophenylmethane sulfonic acid (250 γ/ml.)	24-Hour titer (10^{-n})
—	—	3.8
Postinfection	—	3.8
—	Postinfection	3.2
Preinfection	—	2.5
—	Preinfection	2.2
Pre- and postinfection	—	1.5
—	Pre- and postinfection	1.5
Preinfection	Postinfection	0.8
Postinfection	Preinfection	1.2

* Preinfection treatment started just before inoculation of 10^3 $TCID_{50}$ of virus. After a two-hour adsorption period, cultures were washed six times with double-strength Eagle's basal medium.

effective used preinfection or combined pre- and postinfection. These data further show that inhibition due to either compound is maintained by the other. Although the inhibitory activity of substituted sulfonic acids has been associated with potassium ion,[17] added potassium ion did not reverse either aminophenylamethane sulfonic acid or amantadine hydrochloride in the calf kidney system.

Development of resistance. Since amantadine hydrochloride had been found to inhibit acute rubella virus infection *in vitro*,[5] it was of interest to study its activity against the chronically infected cell line (RA) developed in this laboratory.[8] The results, presented in TABLE 6, show that during four consecutive passages of RA cells chronically infected with rubella virus, treatment with amantadine hydrochloride did not reduce the third week virus yield. Subsequently, the persistence of rubella virus in amantadine-treated, chronically infected cultures was confirmed by immunofluorescence studies. This lack of inhibition in the chronic infection might have resulted from the virus becoming resistant during the prolonged growth period. However, when grown in several consecutive-treated acute infections, with treated virus used as the inoculum for the following passage, rubella virus retained its susceptibility to amantadine, exhibiting the same inhibition as previously untreated virus. Furthermore, when rubella virus from treated, chronically infected cells was used as the inoculum for the acute infection of LLC-MK_2 cells, susceptibility was observed as indicated by a sixth-day virus titer of $10^{-4.7}$ in untreated control cultures.

TABLE 6

COMPARISON OF ACUTE AND CHRONIC RUBELLA INFECTIONS TO INHIBITION BY AMANTADINE HCl *in vitro*

Host cell	Amantadine μg./ml.	$Int._{50}$, 10^{-n}			
		Passage			
		1st	2nd	3rd	4th
RA (chronic)	—	3.5	4.0	4.3	3.7
	31.2	3.0	4.3	4.3	3.5
LLC-MK_2 (acute)	—	4.5	4.3	4.0	4.0
	31.2	2.5	2.7	2.7	2.3

The observed maintenance of the susceptibility of rubella virus to inhibition by amantadine *in vitro* contrasted with the results seen with influenza. When influenza virus was grown in primary calf kidney cells in the presence of amantadine, the development of resistance to inhibition by amantadine was readily apparent, as shown in TABLE 7, where antiviral activity is presented as the therapeutic index.[9] These results show that influenza virus, initially susceptible to amantadine (therapeutic index = 16) or aminophenylmethane sulfonic acid (therapeutic index = 8) in one passage, albeit with several growth cycles, in the presence of either inhibitor lost its susceptibility to both inhibitors.

Discussion

The report by Davies *et al.* on the antiviral activity of amantadine hydrochloride[1] indicated a limited spectrum of activity among the myxoviruses. Their finding that influenza viruses of types A, A_1, A_2, C, and D (Sendai, para-

TABLE 7

DEVELOPMENT OF RESISTANCE TO AMANTADINE HCl AND AMINOPHENYLMETHANE SULFONIC ACID BY INFLUENZA VIRUS (A_2/JAPAN 305) IN PRIMARY CALF KIDNEY CELL CULTURES

	Therapeutic index	
	vs. Amantadine HCl	vs. Aminophenylmethane sulfonic acid
Influenza, Japan 305	16	8
Influenza, Japan 305 passed 1 × with amantadine HCl	1	1
Influenza, Japan 305, passed 1 × with aminophenylmethane sulfonic acid	1	1

influenza), together with the present results, indicate that all types of influenza virus can be susceptible. Our results with the parainfluenza and respiratory syncytial viruses suggest that the resistance of at least certain myxoviruses may be relative rather than absolute, and that susceptibility may not be evident depending on the methods and conditions used. The observed inhibition of infectivity without inhibition of hemagglutination may indicate incomplete virus. Some lines of given strains of influenza virus appear to differ in susceptibility, such as the lines of influenza B-Lee used by Davies *et al.*[1] and by ourselves, as well as similar unpublished experiences of others. However, complicating any consideration of the inherent resistance or susceptibility of viruses to amantadine is the ability of at least one to develop resistance. It is significant that, although influenza virus readily developed resistance *in vitro*, we have yet to demonstrate the emergence of resistance to amantadine *in vivo;* specifically, in treated mice. In any event, amantadine does not engender sufficient resistance to prevent it from being effective *in vivo*, in contrast to guanidine and 2-(α-hydroxybenzyl)-benzimidazole, where resistance does vitiate their effectiveness in monkeys.[18–20]

Our present studies on the locus and mechanism of the antiviral activity of amantadine involve its use in comparison and conjunction with other agents of known activity. The application of X-ray to impair early immune responses indicated that specific antibody was not a necessary complement to amantadine activity *in vivo*. When amantadine was compared *in vitro* to aminophenylmethane sulfonic acid, an inhibitor of influenza virus with a known locus of activity, the two compounds were found to be essentially equivalent and interchangeable in their abilities to inhibit influenza virus and to induce mutual resistance. Neither inhibitor was reversed with selected amino acids.[15]

Rubella virus, in contrast to influenza virus, retained its susceptibility to inhibition by amantadine, even when grown in chronically infected cells where its production was not decreased by amantadine treatment. Amantadine's similarity to aminophenylmethane sulfonic acid, which acts early in the cycle of virus replication during adsorption or penetration,[16] supports the earlier suggestion by Davies *et al.*[1] that amantadine interferes with virus penetration, and is compatible with its decreased effectiveness in delayed treatment against rubella virus *in vitro*.[5] Since the chronic *in vitro* rubella infection was not susceptible, the mode of action of amantadine enables one to conclude that the RA cell line[8] represents a true chronic infection, maintained intracellularly as the cells divide, and not going through an amantadine-susceptible penetration phase. It is tempting to speculate

that the development of resistance to amantadine by influenza virus and the persistence of susceptibility by rubella virus may be related to their immunologic mutabilities—influenza virus having the more variable surface, as reflected by its many and changing serotypes, compared with rubella virus's more stable surface and apparently single serotype.

Amantadine attracted attention through its interesting structure, and seems to be maintaining its attraction in the current laboratory and early clinical testing phases. Its clinical testing requires time and experience, but in any event amantadine is making a significant contribution to the present, accelerating growth of interest and accomplishment, exemplified in part by this symposium, in the chemotherapy and chemoprophylaxis of viral diseases.

Summary

Amantadine hydrochloride at concentrations not toxic to host cells or free virus inhibited the growth of parainfluenza types 2 and 3 and respiratory syncytial viruses *in vitro*.

In mice infected by virus aerosols, protection by amantadine was confirmed against A and A_2 strains and was demonstrated against type B influenza. Vaccinial lung lesions in mice were not inhibited, while in ferrets amantadine treatment aggravated influenza.

Amantadine protected both nonirradiated and irradiated mice and was additive with X-ray in exerting a sparing effect.

The growth of rubella virus in chronically-infected LLC-MK_2 cells was not inhibited by amantadine. This lack of inhibition was not due to the development of resistance by rubella virus, since acute infection of cells with virus produced in treated, chronically infected cells was susceptible. Rubella virus passed repeatedly in acutely infected, treated cells also retained susceptibility to amantadine. In contrast, influenza virus readily developed resistance in treated, acutely infected primary calf kidney cell cultures. When the inhibitory activity of amantadine hydrochloride was compared with aminophenylmethane sulfonic acid, a type of inhibitor acting early in the infectious cycle, both were found similarly effective in suppressing influenza virus production *in vitro* applied preinfection but not post-infection, and that inhibition due to either was maintained equally well by the other. Influenza virus made resistant to amantadine hydrochloride or aminophenylmethane sulfonic acid *in vitro* was also cross resistant to the other. This interchangeability for both inhibition and resistance was interpreted as indicating common sites of activity.

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