JOURNAL OF BACTERIOLOGY, Sept., 1965
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Vol. 90, No. 3
Printed in U.S.A.

Mode of Action of the Antiviral Activity of Amantadine in Tissue Culture[1]

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Received for publication 14 April 1965

ABSTRACT

HOFFMANN, C. E. (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), E. M. NEUMAYER, R. F. HAFF, AND R. A. GOLDSBY. Mode of action of the antiviral activity of amantadine in tissue culture. J. Bacteriol. **90**:623-628. 1965.—Amantadine hydrochloride has shown antiviral activity in tissue culture, in ovo, and in vivo. Experiments with it during the course of virus proliferation indicate that its antiviral activity is due to inhibition of virus penetration into the host cell. These studies indicate that amantadine hydrochloride is not virucidal at concentrations active in tissue culture. It does not block virus adsorption to host cells, nor does it affect the virus enzyme neuraminidase. In the presence of amantadine hydrochloride, virus adsorbed to susceptible cells remains at the cell surface in an infective state. An attempt to demonstrate high development of resistance to the antiviral action of amantadine hydrochloride in tissue culture has been unsuccessful.

Amantadine hydrochloride (1-adamantanamine hydrochloride; Symmetrel, E. I. duPont de Nemours & Co., Inc.) was shown to have antiviral activity in tissue culture and in ovo (Davies et al., 1964; Maassab and Cochran, 1964; Neumayer, Haff, and Hoffmann, 1965). Its antiviral activity in mouse infections was described by Davies et al. (1964) and Grunert, McGahen, and Davies (1965), and in man by Wendel (1964) and Jackson, Muldoon, and Akers (1964). Although antiviral activity was most consistently observed against the A, A1, and A2 strains of influenza virus, other viruses such as rubella, parainfluenza, and pseudorabies were also reported to be sensitive in one or more test systems. Most consistent antiviral activity was obtained with compound added prior to infection. However, in plaque inhibition systems in tissue culture and against certain strains of influenza in mouse infections, continuous treatment with amantadine starting after infection provided a significant antiviral effect.

This communication describes studies in tissue culture systems on the mode of action of amantadine hydrochloride. Direct virus inactivation was not observed at concentrations of compound effective in tissue culture. Neither an inhibition of adsorption of virus to cells nor release of virus from cells was observed, nor was there an effect on the multiplication of intracellular virus. The experiments described indicate that the antiviral activity of the compound is due to a specific block or retardation of virus penetration into the host cell.

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MATERIALS AND METHODS

The materials used in these studies are described by Neumayer et al. (1965). Abbreviations used are as follows: tris(hydroxymethyl)aminomethane (Tris) agar maintenance medium, TAM; TAM with agar omitted, TM; phosphate-buffered saline, PBS; chick-embryo cells, CEC. Stock virus refers to undiluted allantoic fluid from chick embryos infected via the allantoic cavity at day 9 or 10 with the desired virus strain and harvested 40 to 48 hr after infection.

The general methods used were similar to those described by Neumayer et al. (1965), and all modifications are described in the details of specific experiments.

RESULTS

Virus inactivation. A number of virus strains were studied, including ones both sensitive and resistant to amantadine in tissue culture and in vivo. The experiments with influenza A2/Japan/305/57 are typical and were carried out by mixing 50,000 hemadsorption units of virus with 125 and 12.5 μg/ml of amantadine hydrochloride in

maintenance medium. These mixtures, plus a control preparation free from compound, were incubated at 37 C for 24 hr. The initial and residual virus contents were determined by means of a hemadsorption test, and from these determinations the loss of infectivity was calculated. All tests were controlled for residual amantadine in the assay procedure, which was not a problem due to the dilution factor. Tests on other viruses were similar, except that plaque counts were used for the virus assay. The results in Table 1 show the difference in residual infectivity of viruses incubated in the presence and absence of amantadine. No measurable inactivation of virus occurred with 12.5 μg/ml of amantadine, a concentration of compound which provides significant antiviral activity in tissue culture. Under similar conditions, with 100 to 125 μg/ml of amantadine, direct virus inactivation ranged from zero for vaccinia to about 90% for two strains of influenza. However, the two strains most sensitive to direct inactivation, Semliki Forest and vesicular stomatitis, were completely insensitive to inhibition by amantadine in tissue-culture systems and in mouse infections.

Effect of the time of compound addition. Spinner-flask cultures of chick-embryo fibroblasts infected with influenza A2/Japan/305/57 virus were used to study the effect of varying the time of the start of treatment on the antiviral activity of amantadine. Approximately 45 × 10^6 CEC in 17.8 ml of TM medium were placed in each spinner flask and infected with 2 ml of stock allantoic fluid [hemadsorption (HA) titer, 1:1,024]. After a 50-min adsorption period, the cells were precipitated by centrifugation, resuspended in fresh medium, and incubated in the spinner flasks. Amantadine hydrochloride was added to these cultures at a final concentration of 20 μg/ml at different times prior to and after infection and, once added, was present at all times for the remainder of the test. Virus production, determined by HA titration of the cells plus medium, was measured on samples of each culture after incubation periods of 5.75 and 20 hr. A marked inhibition of virus production was observed with treatment starting 10 min prior and up to the time of infection, whereas essentially all antiviral activity was lost when treatment was delayed for 10 min or more after infection (Table 2). The results suggest that the observed effect of compound was on the initial infectious process alone and that only a single cycle of virus multiplication occurred. As reported by Neumayer et al. (1965), delayed treatment with amantadine provided antiviral activity in studies in which multiple cycles of infection occurred.

TABLE 1. *Virucidal activity of amantadine**

Virus	Inactivation (logs)†	
	125 μg/ml	12.5 μg/ml
Influence A/WSN	1.2‡	0.0
Influenza A2/Japan/305/57	1.0	0.0
Vaccinia/WR	0.0	0.1
Vesicular stomatitis/Hazelhurst	2.2	0.5
Semliki Forest	1.6	0.0
Pseudorabies/Aujeszky	0.4	0.0

* As amantadine base neutralized with HCl.

† Increase in loss of virus over a nontreated control held under identical conditions. Incubation was for 24 hr at 37 C.

‡ Value for 100 μg/ml. Log plaque-forming units per milliliter at start, 5.5; incubated control, 3.3; treated series, 2.1; 3.3 − 2.1 = 1.2 log increase in virus destruction over control.

TABLE 2. *Effect of time of amantadine addition on virus production in chick-embryo fibroblasts infected with influenza A2/Japan/305/57*

Time of compound addition*	Negative log of HA titer after incubation for	
	5.75 hr	20 hr
min		
−10	<0.3	<0.3
0†	0.3	0.3
+10	0.9	0.9
+15	1.2	0.9
+30	1.5	1.2
Control	1.2	1.2

* Amantadine hydrochloride (20 μg/ml).

† Added with inoculum.

A second test was carried out to check for possible effects of amantadine on the maturation and release of virus from infected cells. CEC monolayers in petri plates were infected with influenza A2/AA/2/60 virus and overlaid with TM medium. One series of monolayers was treated with 20 μg/ml of amantadine hydrochloride in TM medium during the 3-hr period from 2 to 5 hr postinfection (the period during which detectable virus first appears). Control monolayers were subjected to identical manipulations with TM medium free from compound. At intervals, the cells and fluid from incubated monolayers were harvested separately, and the HA titer was determined on each fraction. Amantadine treatment had no effect on either the maturation of virus or the release of virus from infected cells (Fig. 1).

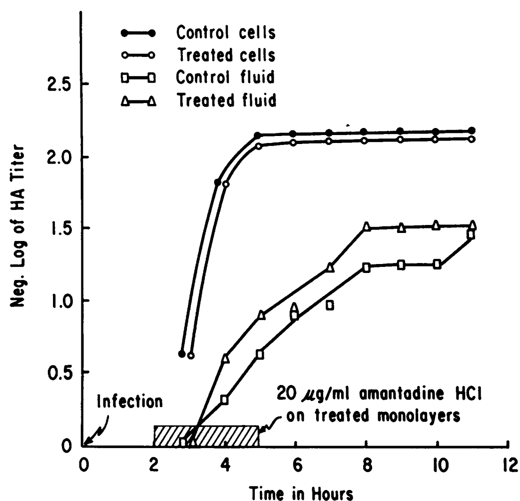

FIG. 1. *Effect of postinfection treatment with amantadine on maturation and release of virus from chick-embryo fibroblasts infected with influenza A2/AA/2/60.*

Effect on adsorption of virus to cells. To determine the effect of amantadine on the adsorption of virus to host cells, samples of a stock suspension of influenza A/WSN virus were added to PBS containing 0, 25, 50, and 100 µg/ml of amantadine hydrochloride. Each of these mixtures was divided into three portions. One was incubated at 4 C, the second at 37 C, and the third was used to inoculate CEC monolayers which were also incubated at 37 C. After 1 hr of incubation, the supernatant fluid from the infected CEC and the other two samples was diluted in PBS and plated on CEC monolayers for plaque assays. Results are shown in Table 3, and, although there is considerable variation, there is no indication of inhibition of adsorption of virus to host cells by amantadine. Other studies with 1, 10, and 100 µg/ml of amantadine measured by the residual HA titer provided similar results.

Effect on virus neuraminidase. Influenza viruses contain an enzyme, neuraminidase, which presumably plays a part in the infectious cycle. Although the exact function of this enzyme is unknown, its action can be studied by means of the release of virus adsorbed to red blood cells. To study the effect of amantadine on this enzyme, a mixture of 13% of an undiluted stock of influenza A2/AA/2/60 virus and 87% of 3.8% chicken red blood cells (RBC) was divided into two portions. One portion received amantadine hydrochloride at 25 µg/ml and was incubated at 37 C. The other portion was divided into two further parts, one of which was incubated at 37 C and the other at 4 C. Samples of these were withdrawn at intervals, centrifuged to remove RBC and adsorbed virus, and the virus content of the supernatant fluid was determined by HA titration. Results of such a test are shown in Fig. 2. There was an initial rapid decrease of the HA titer due to adsorption of virus by the RBC. After this, in samples incubated at 37 C, there was a slow increase in the HA titer of the supernatant fluid which represented release of bound virus due to destruction of the virus binding sites on the RBC by neuraminidase. Although the initial HA

TABLE 3. *Effect of amantadine on adsorption of influenza A/WSN to CEC*

Amantadine hydrochloride	Plaque counts × 10^{-5}			Virus × 10^{-5}*	
	1 hr at 0 C (a)	1 hr at 37 C (b)	1 hr at 37 C + CEC† (c)	Thermal inactivation at 37 C‡ (a − b)	Amt adsorbed (b − c)
µg/ml					
100	30	24	2	6	22
50	32	29	8	3	21
25	25	20	12	5	8
0	28	21	8	7	13

* Virus particles calculated from plaque counts.

† Nonadsorbed virus remaining in the supernatant fluid.

‡ Difference between a and b assumed to be inactivation at 37 C.

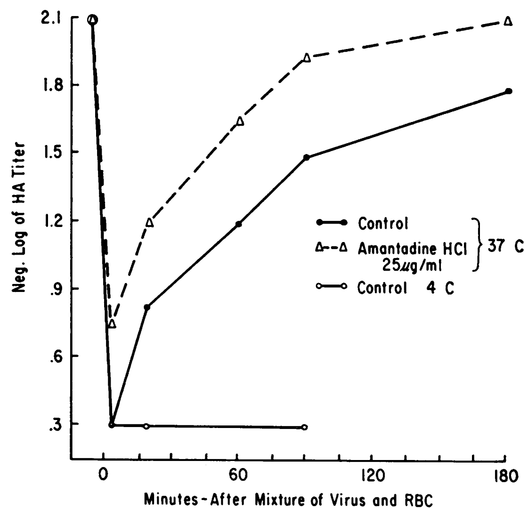

FIG. 2. *Rate of elution of influenza A2/AA/2/60 from RBC in presence or absence of amantadine.*

values at the start of virus release were different, the kinetics of virus release from treated and control cells were similar, indicating little or no effect on this enzyme. No detectable virus was released from cells held at 4 C, which further indicates that the observed virus release at 37 C was enzymatic in nature.

Effect of duration of treatment. Preliminary studies indicated that CEC treated with amantadine hydrochloride for as long as 4 hr and then washed with PBS would support normal multiplication of virus when subsequently infected. This finding provided a method for the determination of the effect of varying durations of treatment on virus production. Amantadine hydrochloride was added to CEC monolayers at 20 μg/ml 15 min prior to infection. A 1:10 dilution of an allantoic fluid stock (HA titer, 1:512) of influenza A2/AA/2/60 virus was used as the inoculum with a 20-min adsorption period. Once added, compound was present at all times until removed by washing the cell monolayers with PBS. The virus content of the cell monolayers was determined by HA titration of frozen and thawed cells. Results of tests with 2 and 3 hr of compound treatment are shown in Fig. 3, as well as those obtained from untreated controls. There was a direct correlation between the first appearance of virus within the cells and the length of treatment. The total amount of virus produced was also correlated with the length of treatment time. Prolonged incubation resulted

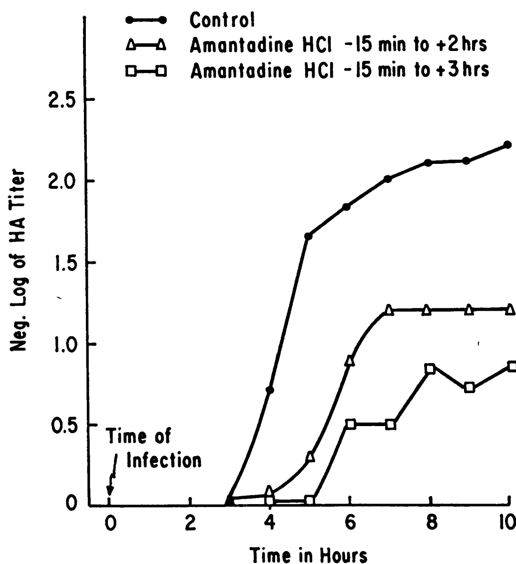

FIG. 3. *Effect of length of treatment time on virus production of CEC infected with influenza A2/AA/2/60.*

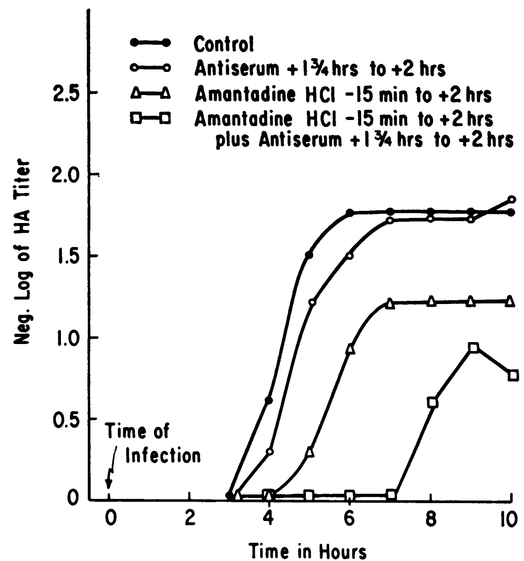

FIG. 4. *Effect of amantadine, antibody, and combinations on the virus production of CEC infected with influenza A2/AA/2/60. HA titrations were carried out on frozen-thawed cells.*

in slightly more virus in both treated and control cultures, but the amount in treated cultures never reached that of controls. It is possible that compound was not completely removed in the presence of virus, or that a certain amount of the virus inoculum was inactivated during the incubation period prior to removal of compound.

Fate of virus adsorbed to treated cells. In a report by Davies et al. (1964), it was shown that virus adsorbed to cells in the presence of amantadine was susceptible to inactivation by antibody several hours after infection, whereas virus in a corresponding compound-free system was not. Further studies have been carried out with CEC monolayers infected with influenza A2/AA/2/60. After a 15-min incubation period, unadsorbed virus was removed by washing the monolayers with PBS. Compound-treated monolayers received 20 μg/ml of amantadine hydrochloride for the period from 15 min prior to 2 hr after infection, and both the inoculum and PBS wash also contained 20 μg/ml of compound. Antibody-treated monolayers received a 1:160 dilution of specific virus rabbit antiserum for a 15-min period between 1.75 and 2 hr postinfection. The production of virus, as measured by HA titration of frozen-thawed cell layers, during the 3- to 10-hr postinfection period is shown in Fig. 4. Antiserum alone provided no determinable inhibition of virus production when added during the indicated period. Amantadine alone

produced the usual delay in initial appearance of virus as well as the usual reduction in total amount of virus. However, the combination of antiserum plus compound resulted both in a marked delay in appearance of virus as well as a marked reduction in total virus produced. This confirms the previous work and provides further evidence for the concept that, in the presence of amantadine, adsorbed virus remains at the cell surface in an infectious state.

Further confirmation of this observation was obtained by use of hemadsorption techniques. Cells infected with influenza virus and immediately treated with chicken RBC will adsorb the RBC. However, when infected cells are incubated at 37 C for short periods prior to addition of RBC, the adsorbed virus penetrates into the cells and RBC added subsequently are no longer bound. Incubation at 3 to 4 C prevents virus penetration and infected cells retain their RBC binding capacity. In a preliminary study with amantadine, 2 ml of CEC containing 9 × 10^6 cells per milliliter in PBS were mixed with 2 ml of influenza A/NWS (500 hemagglutination units per milliliter) at 0 C. Unadsorbed virus was removed by washing after 5 min; the washed cells were resuspended in 4 ml of PBS and divided into four portions. Two portions received 100 μg/ml of amantadine hydrochloride, and two were untreated. One set consisting of treated and untreated cells was incubated at 37 C, and the second at 0 C. After 45 min of incubation at 37 C, the ability to adsorb RBC was determined by adding 0.5 ml of 0.5% chicken RBC to each set and determining the agglutination score. Both treated and untreated cells at 0 C gave a score of 4+ as compared with a score of 3+ for treated and + for untreated cells incubated at 37 C.

A more quantitative test was carried out by the use of CEC monolayers infected with influenza A2/Japan/305 virus. Treated monolayers received 20 μg/ml of amantadine hydrochloride (present throughout once added) 15 min prior to infection with 1 ml of a 1:2 dilution of virus with an HA titer of 1:1,024. Virus was removed by washing after 15 min, and the washed monolayers were incubated at the desired temperatures for 30 min. All monolayers were placed at 4 C, and 3 ml of chicken RBC (0.5%) in PBS were added. After 15 min, unadsorbed RBC were removed by washing, and the adsorbed RBC were allowed to elute into 3 ml of PBS during a 45-min incubation period at 37 C. The optical density of the eluted RBC was determined at 540 mμ. Infected monolayers incubated at 37 C in the absence of amantadine lost their ability to bind RBC, whereas treated cells retained the capacity to bind RBC (Table 4). There was little difference between treated and untreated monolayers incubated at 4 C. These tests indicate that, in the presence of amantadine hydrochloride, adsorbed virus is retained at the cell surface.

TABLE 4. *Effect of amantadine on adsorption of red blood cells to virus-infected incubated chick-embryo fibroblast monolayers*

Test conditions			Optical density at 540 mμ
Incubation temp	Amantadine, 20 μg/ml	Virus	
C			
37	+	−	0.00
37	−	+	0.065
37	+	+	0.225
4	−	+	0.460
4	+	+	0.500

Development of virus resistance. In plaque-inhibition studies with influenza A/NWS virus, plaques occasionally appear within the zone of inhibition produced by amantadine. The virus from one such plaque was isolated and serially passed eight times in CEC monolayers in the presence of 25 μg/ml of amantadine. Virus from the final transfer was used to infect CEC monolayers at a dilution which provided 100 plaques per monolayer. The monolayers were treated with amantadine, and, after suitable incubation, the number of plaques was counted. At 4 μg/ml, the passaged strain showed no reduction in the number of plaques as compared with untreated controls, whereas the parent strain showed a 10% reduction. With 20 μg/ml, the per cent reduction in plaque number was 12 for the passaged strain and 18 for the parent strain. Under these conditions, it appears that a marked development of resistance to amantadine did not occur. Similar results were obtained by Grunert et al. (1965) with eight serial passages of influenza A/swine/S15 through mice treated with amantadine hydrochloride. These observations may be a reflection of the test conditions used since Cochran et al. (1964) reported that an A2 strain of influenza propagated in primary calf kidney cells rapidly developed resistance to amantadine. This was not observed with passage of virus in mice treated with amantadine hydrochloride nor with rubella virus passaged in LLC MK_2 cells.

DISCUSSION

The tissue-culture studies described indicate that amantadine specifically blocks the penetration of sensitive strains of virus into the host

cells. Adsorption of virus to the cell surface is not inhibited, but once adsorbed on the cell surface the virus remains attached in an infectious form. This mode of action is unique among antiviral agents acting in vivo, although the end results in tissue culture resemble those observed with specific virus antibody. However, antibody reacts directly with the virus, whereas the activity of amantadine does not appear due to a direct action on the virus.

The antiviral effect of amantadine appears to be mediated through reactions with the host cell, but the specificity of action observed is virus- and not host system-related. Possibly the compound inhibits an enzyme involved with virus penetration into host cells. If this is the case, results obtained to date would indicate that such an enzyme must be in all cells supporting virus multiplication, and also that different viruses require different enzymes, only some of which are inhibited by amantadine. Alternatively, it is possible that the viruses sensitive to amantadine penetrate by means of a cell enzyme, and that insensitive viruses penetrate by some other mechanisms. Another possibility is that amantadine has no direct effect on virus penetration but, instead, acts by binding sensitive strains of virus on areas of the cell surface (if these actually exist) not susceptible to penetration. The net effect of such a mechanism would be to lower the number of virus particles reaching penetration sites and thus reduce the amount of infection. However, since the present knowledge of the mechanisms of virus penetration is incomplete, these are purely speculations which may have little to do with the actual mode of action of this compound.

The data obtained with amantadine suggest its use for a number of applications. In the laboratory, amantadine may be of value both as a tool for studying mechanisms of virus penetration and as an aid in virus classification. However, of greatest importance is the demonstration that inhibition of virus penetration into the host cell is a practical target for the prevention of virus infections, as has been demonstrated in the reports of antiviral activity in a variety of host systems including man.

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