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Anti-Influenza A Virus Activity of Amantadine Hydrochloride and Rimantadine Hydrochloride in Ferret Tracheal Ciliated Epithelium

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The activities and toxicities of amantadine hydrochloride and rimantadine hydrochloride against influenza A/Alaska/6/77 (H3N2) and A/Bangkok/1/79 (H3N2) viruses were compared in organ cultures of ferret tracheal ciliated epithelium. Pretreatment of cultures with concentrations (0.5 and 1 μg/ml) comparable to those found in human serum after oral administration of amantadine revealed that rimantadine produced significantly longer protection than amantadine against virus-induced cytopathic effects. Correspondingly, rimantadine produced a comparable protective effect at four- to eight-fold-lower concentrations than amantadine. Both drugs produced increasing and similar effects at higher concentrations, which were comparable to those reported in nasal washings after aerosol administration of amantadine. At the concentrations tested, amantadine was nontoxic. However, at concentrations of 16 and 32 μg/ml, rimantadine was toxic to the ciliated epithelium after 10 to 21 days of continuous exposure. When the drugs were added 24 h or more after infection, protection against cytopathic effects decreased markedly. Both drugs moderately suppressed virus production at concentrations of 0.5 to 16 μg/ml. However, no dose response or difference between the drugs was observed. Because of comparable antiviral activity at lower concentrations and greater activity at similar concentrations, rimantadine may be more useful than amantadine for oral prophylaxis and therapy of influenza.

Amantadine hydrochloride has been evaluated widely as a chemoprophylactic agent against influenza A virus (6, 17, 18) and has established efficacy for preventing or modifying disease (22, 24, 25, 27). As a therapeutic agent, amantadine hastens resolution of uncomplicated influenza (8, 36), but in established influenza pneumonia less impressive results have been observed (7).

To increase the clinical efficacy of amantadine, three approaches have been used. One approach has been the use of larger oral doses; however, dose-dependent side effects make significant increases unacceptable (4, 13). A second approach has been the development of amantadine analogs (27). One of these, rimantadine hydrochloride, has been reported to be more active than amantadine in vitro and at least as active as amantadine in clinical trials (11, 28, 33). However, there are conflicting (18, 28, 33, 36) in vitro reports (11, 32).

The third approach has been to increase the concentration of amantadine at the target tissue of influenza infection (the respiratory ciliated epithelium) by aerosol delivery. Amantadine aerosol has been effective when administered to volunteers for therapy (14). However, the inconvenience of multiple daily treatments totaling several hours limits this method of administration. In addition, it has produced local side effects (15), possibly caused by direct toxicity of amantadine to ciliated cells (5).

Arroyo and Reed used the ferret tracheal organ culture model to compare spiroamantadine, cycloamantadine, and amantadine and correlated the in vitro effects with antiviral activity in humans (2, 3). These authors observed the ciliated epithelium for cytopathic effects (CPE) but presented their results only in terms of drug suppression of virus replication. The ability of the drugs to protect the ciliated epithelium against virus-induced CPE was not discussed. Furthermore, Arroyo and Reed used relatively large virus inocula (10^5 50% egg infective doses) and drug concentrations that were many times greater than attainable serum levels.

The tracheal organ culture model would be more useful if the anti-influenza effect could be

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demonstrated at concentrations of amantadine found in serum after oral administration (0.2 to 1.3 μg/ml) (9) and with virus inocula near the human 50% infective dose (3 50% tissue culture infective doses) when influenza virus is administered by aerosol (1, 20). There is an inoculum effect on the anti-influenza activity of amantadine in mice and in tissue culture (the drug is more active at low inocula) (10, 12). Therefore, low inocula should permit measurement of antiviral activity at lower drug concentrations. Accordingly, we evaluated the relative anti-influenza activities of amantadine and rimantadine in ferret tracheal organ cultures at drug concentrations which are achievable in serum and with low virus inocula. In addition, we evaluated the relative potencies of these drugs at higher concentrations (up to the limits of direct toxicity).

MATERIALS AND METHODS

Organ culture. Using the method of Mostow and Tyrrell, we dissected tracheal rings from 6- to 10-week-old ferrets (23). Each ring was placed in a screw-capped tube containing 1 ml of Liebowitz 15 medium (L15) (19) supplemented with 100 U of penicillin and 20 μg of gentamicin and incubated at 37°C in a roller drum. Since a preliminary experiment showed no difference in ciliary survival in infected rings treated with 1 μg of amantadine per ml when the medium was changed twice weekly instead of daily (data not shown), the medium was replaced on days 1, 3, 7, 10, 14, 17, and 21.

Assessment of ciliary activity. A single observer estimated the fraction of the circumference of each ring with actively beating cilia and recorded this as the percentage of the base-line value. The observer did not know to which experimental condition each ring belonged. A score ($T_{1/2}$) was assigned to each ring based on the number of days from initiation of infection until the ciliary activity dropped below one-half of the base-line activity (interpolated to the nearest 0.1 day) (16). For some drug concentrations, the $T_{1/2}$ could not be calculated because the ciliary activity of some or all rings persisted at more than one-half of the base-line value throughout the experiment. Statistical comparisons were performed only among groups in which all rings had a $T_{1/2}$ of 21 days or less. Each group contained four rings in separate tubes, and each experiment was repeated two or three times with tracheal rings from different animals. Concurrent infected and uninfected control groups were included in each experiment.

Virus. Influenza A/Alaska/6/77 (H3N2) virus in egg passage 13 and influenza A/Bangkok/1/79 (H3N2) virus also in egg passage 13 were titrated in ferret tracheal rings to determine their 50% infective doses. For each virus this was found to be 3 50% tissue culture infective doses (in Madin-Darbey canine kidney cells). To ensure that every ring was infected, each ring was inoculated with 10 to 32 times this amount, except where specified otherwise. The ring was then allowed to adsorb virus for 1 h at 37°C, washed three times in L15, and incubated at 37°C in a roller drum.

Drugs. For each experiment, amantadine hydrochloride and rimantadine hydrochloride (both kindly provided by John LaMontagne, National Institute of Allergy and Infectious Diseases) were weighed, dissolved in L15 to the appropriate concentrations, and sterilized by membrane filtration (Millipore Corp.). Each drug was tested for activity against influenza A/Alaska/6/77 virus at doubling concentrations from 0.5 to 32 μg/ml. In addition, amantadine was tested for activity against influenza A/Bangkok/1/79 virus at concentrations of 0.5, 1, and 16 μg/ml, and rimantadine was tested for activity against this virus at concentrations of 0.06, 0.125, 0.25, 0.5, 1, and 16 μg/ml. In preinfection experiments, L15 supplemented with dissolved drug was added to the rings 2 h before infection and with each wash and medium change. In postinfection experiments, which were performed only with influenza A/Alaska/6/77 virus, drug-free medium was first replaced by drug-containing medium at 2, 6, 24, 36, 48, or 60 h after initiation of viral infection. Controls for drug toxicity were included at concentrations of 8 μg/ml and greater.

Virus titration. During the experiment the medium from each group of rings was harvested in toto on the days indicated below. This medium was then mixed with an equal volume of Hanks balanced saline solution containing 3 mg of bovine serum albumin per ml as a stabilizer and stored at −70°C.

The infectivity titers of pooled samples from each group were determined in 5-day monolayers of Madin-Darby canine kidney cells in microtiter plates; 200 μl of L15 containing 1 μg of crystaline trypsin per ml was added to each well. Each sample was serially diluted in 10-fold steps in triplicate. After inoculation and 96 h of incubation at 37°C, 50 μl of 0.5% washed guinea pig erythrocytes was added to each well at room temperature, and hemabsorption endpoints were determined 0.5 to 1 h later. The titer was calculated by the method of Reed and Muench (29).

RESULTS

Uninfected control rings maintained 70 to 100% of the base-line activity for at least 21 days. In the initial experiments with influenza A/Alaska/6/77 virus, the ciliary activity of the infected epithelium had a $T_{1/2}$ of 3.3 ± 0.5 days (mean ± standard deviation for 24 rings). Pretreatment with amantadine at doubling concentrations showed a dose-dependent effect; the $T_{1/2}$ was 5.7 days at a concentration of 0.5 μg/ml and 18.9 days at a concentration of 32 μg/ml (Table 1). Rimantadine was also tested at doubling concentrations ranging from 0.5 to 32 μg/ml, and this drug delayed the $T_{1/2}$ longer at the lower concentrations and nearly the same at the higher concentrations. Thus, there was a less marked dose-dependent effect.

As Table 1 shows, at drug levels comparable to those in sera of patients receiving oral therapy (0.5 and 1 μg/ml), rimantadine was more active than amantadine. The differences were significant at the 0.05 level as determined by the two-tailed Wilcoxon rank sum test (35). With both drugs, increasing the concentration prolonged the $T_{1/2}$; however, there were decreasing differ-

TABLE 1. Effects of amantadine and rimantadine on CPE induced by influenza A/Alaska/6/77 virus[a]

Drug concn (μg/ml)	T½ (days) with:	
	Amantadine	Rimantadine
0.5	5.7 ± 0.8[b]	11.7 ± 1.9
1	6.9 ± 1.7	11.7 ± 2.3
2	7.6 ± 1.6	12.3 ± 1.9
4	10.4 ± 2.6	13.6 ± 2.8
8	13.7[c]	14.7[c]
16	18.5[c]	18.4[c]
32	18.9[c]	9.7 ± 1.3

[a] For virus-infected ciliated epithelium with no drug added the T½ was 3.3 ± 0.5 days. The results are expressed as mean ± standard deviation (n = 12).
[b] At P = 0.05 the difference is significant, as determined by the two-tailed Wilcoxon rank sum test (35).
[c] Some rings showed <50% CPE on day 21.

TABLE 3. Effects of amantadine and rimantadine on CPE induced by influenza A/Bangkok/1/79 virus[a]

Drug concn (μg/ml)	T½ (days) with:	
	Amantadine	Rimantadine
0.06	ND[b]	6.27 ± 1.5
0.12	ND	8.4 ± 1.9
0.25	ND	8.7 ± 3.7
0.5	5.7 ± 0.16[c]	12.4 ± 1.94
1	6.5 ± 1.6[c]	11.7 ± 2.73
16	10.7 ± 2.1	10.1 ± 2.4

[a] For virus-infected ciliated epithelium with no drug added the T½ was 3.8 ± 0.4 days. The results are expressed as mean ± standard deviation (n = 8).
[b] ND, Not done.
[c] At P = 0.05 the difference is significant, as determined by the two-tailed Wilcoxon rank sum test (35).

ences between the compounds. A drug-induced toxic effect was observed with rimantadine at concentrations of 16 and 32 μg/ml (Table 2). This explains the low $T_{1/2}$ for rimantadine at a concentration of 32 μg/ml in Table 1; 8 μg of rimantadine per ml and 32 μg or less of amantadine per ml caused no drug-induced toxic effects during the 21-day experiment.

The results described above were inoculum dependent. When the inoculum of influenza A/Alaska/6/77 virus was increased 1,000-fold, pretreatment with either drug at a concentration of 1 μg/ml or even 16 μg/ml delayed the $T_{1/2}$ by less than 2 days compared with infected controls.

When the preinfection observations were extended to a second H3N2 virus, influenza A/Bangkok/1/79 virus, similar results were obtained at concentrations of 0.5 and 1 μg/ml; rimantadine protected the ciliated epithelium significantly longer than amantadine (Table 3). The activity of rimantadine against this virus was also checked at lower concentrations. Protection against CPE comparable to that produced by 0.5 to 1 μg of amantadine per ml was found with 0.06 to 0.12 μg of rimantadine per ml. At 16 μg/ml, a concentration at which rimantadine produced the maximal therapeutic effect (Table 1) but also produced late toxicity, the durations of protection were similar for the two drugs.

To simulate therapy of established infections as opposed to prophylaxis, drugs were added at increasing times after initiation of infection with influenza A/Alaska/6/77 virus. This resulted in a decrease in the duration of protection (Table 4). This was most marked at times greater than 24 h. At drug concentrations shown to be effective when drugs were added before initiation of infection (8 and 16 μg/ml), no significant differences between the drugs were observed, although there was a modest dose-dependent effect for amantadine. In a single experiment, rimantadine at a concentration of 1 μg/ml added 24 h after virus inoculation provided less than 0.5 day of protection against CPE (compared with infected control rings). This is in contrast to

TABLE 2. Drug toxicity to uninfected ciliated epithelium

Drug concn (μg/ml)	Time (days) until 50% destruction	
	Amantadine	Rimantadine
8	≥21[a]	≥21[a]
16	≥21[a]	17.8 ± 2.3[b]
32	≥21[a]	11.2 ± 4.4[b]

[a] After 21 days the experiment was discontinued (all rings had >50% intact ciliated epithelium).
[b] Mean ± standard deviation (n = 8).

TABLE 4. Effects of drugs on CPE induced by influenza A/Alaska/6/77 virus when drugs were added at increasing times after infection[a]

Time drugs added (h)	T½ (days) with:			
	Amantadine		Rimantadine	
	8 μg/ml	16 μg/ml	8 μg/ml	16 μg/ml
−2	13.7[b]	18.5[b]	14.7[b]	18.4[b]
+2	16.0[b]	17.9[b]	15.0[b]	12.1 ± 5.1
+6	11.3 ± 3.2	15.7[b]	14.2 ± 3.7	11.6 ± 3.2
+24	9.4 ± 2.4	17.1[b]	10.7 ± 2.3	14 ± 4.8
+36	ND[c]	7.2 ± 2.3	ND	6.3 ± 2.5
+48	ND	5.0 ± 1.7	ND	5.1 ± 1.6
+60	ND	3.9 ± 0.6	ND	4.1 ± 0.7

[a] For virus-infected ciliated epithelium with no drug added the T½ was 3.34 ± 0.48 days. The results are expressed as mean ± standard deviation (n = 8).
[b] Some rings showed <50% CPE on day 21.
[c] ND, Not done.

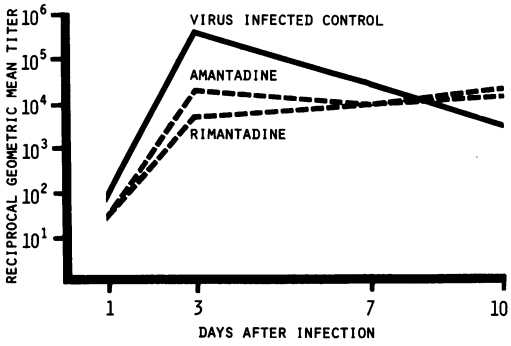

FIG. 1. Production of virus in influenza A/Alaska/6/77 (H3N2) virus-infected ferret tracheal ciliated epithelium: drug-free controls compared with ciliary epithelium pretreated with and maintained in the presence of 1 μg of amantadine hydrochloride per ml or 1 μg of rimantadine hydrochloride per ml. Titer on each day marked is the geometric mean of three titrations.

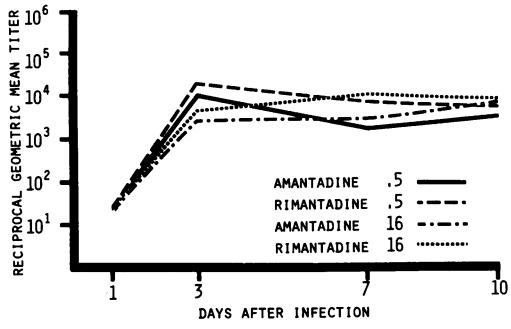

FIG. 2. Production of virus in influenza A/Alaska/6/77 (H3N2) virus-infected ferret tracheal ciliated epithelium: comparison of the effects of pretreatment with 0.5 and 16 μg of amantadine hydrochloride per ml and 0.5 and 16 μg of rimantadine per ml. Titer on each day marked is the geometric mean of three titrations.

the pretreatment effect at this concentration. Because of the inoculum effect noted above, this was expected and not pursued.

When the pooled media were titrated for the amount of virus produced, infected control groups produced measurable virus titers on day 1 and peak virus titers on day 3, with a progressive decrease thereafter, as shown for influenza A/Alaska/6/77 virus (Fig. 1). Drug-treated groups showed 10- to 100-fold suppression of peak virus titers and flattening of the curve. No clear dose response and no apparent differences between amantadine and rimantadine were observed (Fig. 2). Similar virus titer changes were observed after drug treatment of both viruses tested. In the postexposure experiments with influenza A/Alaska/6/77 virus, the effect on virus production decreased over time. When treatment was initiated 48 h or more after inoculation, virus production by the drug-treated groups was similar to the production by the virus controls.

DISCUSSION

In ferret tracheal organ cultures, wild-type influenza A viruses kill ciliated cells, as they do in severe infections in intact ferrets (8) and humans (21). In this study we found that both amantadine and rimantadine delay damage to influenza A (H3N2) virus-infected ciliated epithelium. When drugs are added before infection at concentrations ranging from 0.5 to 2 μg/ml, rimantadine is more effective than amantadine. These levels are comparable to the levels achieved in sera after oral administration of 200 mg of amantadine per day to humans (9), a dose regimen which has been shown to be effective for prophylaxis against influenza (22, 24, 25, 27). Furthermore, rimantadine produced protection against CPE roughly equal to the protection produced by 0.5 to 1 μg of amantadine per ml at eightfold-lower concentrations.

After oral administration, the dose-limiting side effects observed are neurological and gastrointestinal and occur at similar doses of amantadine and rimantadine (4, 13). Therefore, these data predict a severalfold-greater therapeutic index for orally administered rimantadine.

When CPE are measured, both amantadine and rimantadine show increasing antiviral activity as concentrations are increased. Amantadine at a concentration of 32 μg/ml produced no toxic effect, whereas rimantadine at concentrations of 16 and 32 μg/ml was toxic during weeks 2 and 3. When amantadine is administered to humans by aerosol, some patients show respiratory side effects (15). At 1 h after aerosol administration, nasal concentrations of 30 μg/ml have been reported (15). Thus, although levels of drug which produce increased antiviral effects may be achieved by aerosol administration, the greater potential toxicity of rimantadine may preclude its use by this route.

To simulate treatment of established infections, the drugs were added at increasing times after virus inoculation. This produced a diminished drug effect with increasing intervals.

Host defenses, such as mucous clearance, humoral factors, and cellular factors, are absent in this model. This may explain why in intact animals both amantadine and rimantadine are effective later after the initiation of infection (30, 34) than observed in this study.

We found a discrepancy between the delay in CPE and virus replication. Even at drug concentrations where the $T_{1/2}$ was delayed to more than 18 days, virus replication on day 3 was suppressed only moderately. Furthermore, there

was no clear relationship between the dose of drug or which drug was used and suppression of virus replication. Virus-induced CPE (i.e., damage to ciliated cells) correlate well with the spread of infection through the epithelium as assessed by immunofluorescence (S. Mostow and B. Murphy, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 17th, abstr. no. 277, 1977). In the presence of drugs the virus eventually produced widespread CPE; therefore, essentially every cell must have been infected. In the groups treated with 0.5 and 1 μg of amantadine per ml, widespread CPE occurred, whereas virus production was moderately suppressed. In the group treated with 16 μg of amantadine per ml on days 7 to 18 after infection, no CPE were observed, and virus production was similarly suppressed. This suggests that in the presence of either drug, virus-host cell interactions are altered in such a way as to inhibit virus production partially. Over the concentration range tested, this effect is apparently independent of drug-mediated changes in cytopathic activity.

The relative importance of virus replication and damage to the ciliated epithelium in the pathogenesis of influenza is unknown (31). However, it seems likely that epithelial damage is important in influenza-associated airway dysfunction. In previous reports on the relative in vitro anti-influenza activities of amantadine and rimantadine, only the effects of replication of influenza A virus have been described (3). We have shown that drugs differ in ability to protect the ciliated epithelium in vitro. If protection against CPE is important independent of virus suppression, then rimantadine may offer important advantages over amantadine. Although low concentrations similarly suppress virus production, they cause considerably longer delays in CPE. At higher concentrations, such as those that may be achieved after aerosol administration, amantadine is as active as and less toxic than rimantadine. Interpretation of these in vitro observations will depend upon appropriate in vivo and clinical studies.

The ferret tracheal culture model of influenza virus infection permits in vitro comparison of antiviral drugs. Protection against virus-induced CPE, reduction in viral replication, and toxicities of compounds can be evaluated readily with this system.

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