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Plaque Inhibition Assay for Drug Susceptibility Testing of Influenza Viruses

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The relative antiviral activities of four drugs against contemporary strains of influenza A and B viruses were determined in Madin-Darby canine kidney cell monolayers with a plaque inhibition assay. This assay proved to be a reliable, rapid method of determining 50% inhibitory concentrations that correlated well with clinically achievable drug levels and the results of clinical trials. Contemporary strains of influenza A viruses (subtypes H1N1, H3N2, HSW1N1) required amantadine hydrochloride and rimantadine hydrochloride 50% inhibitory concentrations in the range of 0.2 to 0.4 μg/ml, whereas 50% inhibitory concentrations ranged from approximately 50 to 100 μg/ml against influenza B viruses. Ribavirin was approximately 10-fold less active than amantadine hydrochloride against influenza A viruses, and the ribavirin 50% inhibitory concentrations against both influenza A and B viruses ranged from 2.6 to 6.8 μg/ml. Inosiplex had no antiviral activity in this test system.

Various compounds have been reported to possess antiviral activity for influenza viruses in vitro and in animal models of influenza virus infection. However, only amantadine hydrochloride and the closely related derivative rimantadine hydrochloride, have documented prophylactic and therapeutic effectiveness in naturally occurring influenza A virus infections of humans (22, 40; L. P. Van Voris, F. G. Hayden, R. B. Betts, R. G. Douglas, Jr., and W. A. Christmas, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., abstr. no. 483, 1978). Amantadine hydrochloride has thus become the standard against which other anti-influenza compounds are compared. Rimantadine hydrochloride has been reported to be more active than amantadine both in vitro (35, 39) and in vivo (29, 39). In addition, two unrelated compounds, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) and inosiplex (isoprinosine), have been reported to be active against various viruses, including influenza A and B viruses (5, 16, 23, 24).

In the United States, amantadine was initially licensed for prevention of infection due to H2N2 subtype influenza A virus in 1965. Influenza due to H3N2 subtype began with the A/Hong Kong pandemic of 1968-1969, and epidemics due to related H3N2 strains continued for the next decade. However, amantadine was not licensed by the Food and Drug Administration for use in infections due to the H3N2 subtype of influenza A virus until 1976. This delay was in part related to the necessity to perform field trials of amantadine in infections due to the different subtypes of influenza A viruses. This experience highlights the need for standardized methods for predicting the antiviral activity of amantadine and other antiviral agents against recent influenza isolates.

The purpose of the present study was to develop a rapid, reliable assay for determining the susceptibility of influenza viruses to available antiviral drugs and to compare the anti-influenza activity of these drugs against recent isolates. Unlike previous studies, we sought to determine whether antiviral activity based on in vitro susceptibility testing could be related to achievable blood or tissue levels of the drugs and, further, to the results of clinical trials.

MATERIALS AND METHODS

Drugs. Crystalline powders of amantadine hydrochloride and rimantadine hydrochloride were kindly provided by E. I. Du Pont de Nemours and Co., Wilmington, Del.; ribavirin was provided by ICN Pharmaceuticals, Inc., Covina, Calif.; inosiplex was provided by Newport Pharmaceuticals, Inc., Newport Beach, Calif.; and rifampin was provided by Dow Chemical Co., Indianapolis, Ind. Rifampin, which has in vitro inhibitory activity against certain deoxyribonucleic acid viruses but not ortho- or paramyxoviruses (15, 33), was included as a negative control in certain experiments.

Immediately before each experiment, drugs were solubilized in sterile, double-distilled water, and dilutions were made in cell culture medium (below). Rif-

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ampin was initially made up in methanol at a concentration of 10 mg/ml and subsequently diluted in distilled water and medium.

Cell culture. Madin-Darby canine kidney (MDCK) cells (Flow Laboratories, Inc., Rockville, Md.) were passaged weekly with growth medium consisting of Eagle minimal essential medium, glutamine, 10% heat-inactivated fetal bovine serum, penicillin, and gentamicin.

For drug susceptibility testing (below), disposable 35-mm plastic culture dishes (Costar, Cambridge, Mass.) were seeded with approximately 10^5 MDCK cells in 2.0 ml of growth medium and incubated with 5% CO_2 at 36°C for 3 to 5 days, until confluent monolayers had grown.

Viruses. Seven strains of influenza A viruses, representing four subtypes (H0N1, H1N1, HSW1N1, H3N2), and four strains of influenza B viruses were used in these studies. The influenza A viruses were A/PR/8/34/H0N1, A/USSR/90/77/H1N1, A/Fukushima/78/H1N1, A/Brazil/78/H1N1, A/New Jersey/76/HSW1N1, A/Victoria/3/75/H3N2, and A/Texas/1/77/H3N2; the influenza B viruses were B/Maryland/72, B/Hong Kong/76, B/Leningrad/235/74, and B/Rochester/77. In addition, 12 isolates of B/Rochester/77 and 4 isolates of A/USSR/90/77/H1N1 obtained during natural outbreaks were studied. Except for the highly passaged A/PR/8 strain, these viruses were clinical isolates either recovered in the University of Rochester Influenza Surveillance Laboratory or provided by Allan Kendall, Virology Division, Center for Disease Control, Atlanta, Ga. The University of Rochester isolations were initially made in primary rhesus monkey kidney cell monolayers (Microbiological Associates, Bethesda, Md.). Virus stocks were prepared in embryonated hen's eggs (1 to 14 passages), and samples of allantoic fluid were stored at −70°C. For susceptibility tests, virus dilutions were made in Hanks balanced salt solution (pH 7.2 to 7.4) containing 0.5% gelatin.

Plaque inhibition assay. Drug susceptibility tests were performed with modifications of methods described by Kremzner and Harter (18) and by Tobita et al. (36). Triplicate monolayers of MDCK cells in 35-mm culture dishes were washed free of protein-containing growth medium before use and preincubated with 0.2 ml of doubly concentrated Eagle minimal essential medium (pH 7.2 to 7.4) containing trypsin (4 μg/ml; Worthington Biochemicals Corp., Freehold, N.J.) and the test drug in double concentration. An equal volume of virus suspension, containing 50 to 150 plaque-forming units, was added 5 to 10 min later, and plates were incubated at 36°C for 60 min with frequent shaking. A 0.6% agarose overlay (3 ml) containing Eagle minimal essential medium, trypsin (2 μg/ml), and the appropriate drug dilution was added to each plate. Plates were incubated at 36°C in an humidified atmosphere of 5% CO_2 in air. After 36 to 48 h, plaques were stained with neutral red and counted. Depending on the test drug, the final concentrations in the overlay ranged in doubling or fourfold dilutions from 0.025 to 200 μg/ml. The percentage of plaque inhibition relative to the infected control (no drug) plates was determined for each drug concentration, and the 50% inhibitory concentration (IC_{50}) was calculated by probit analysis.

RESULTS

Plaque assay method. Initial studies determined that the number of plaques depended both on the duration of the adsorption period before the agarose overlay and on the presence or absence of drug during the adsorption period. In three experiments, the average number of plaques decreased by 12 to 19% when the adsorption period decreased from 60 to 30 min ($0.05 < P < 0.1$; t test), and increased by 0 to 12% when the adsorption period was increased to 120 min ($P > 0.1$; t test). When low concentrations of rimantadine were incorporated in the maintenance media during the virus adsorption period, the number of A/Texas/77/H3N2 plaques decreased by 26% (30 min), 47% (60 min), or 38% (120 min), respectively, relative to the number observed when the drug was only present in the agarose overlay ($P < 0.001$; t test). Previous studies have shown that the antiviral activity of amantadine is abolished when the drug is added to cell cultures later than 30 min after challenge with a high multiplicity of virus (30). Thus, in subsequent experiments the test drug was present in the fluid overlay during the 60-min virus adsorption at a concentration equivalent to that present in the agarose overlay. Other preliminary studies showed that the brief use of a hypertonic instead of an isotonic fluid overlay during viral adsorption did not affect the number of plaques.

The reproducibility of this assay was assessed by determining sequential drug susceptibility results for an influenza A and an influenza B virus in five separate assays over a 6-month period. The mean (± standard deviation) amantadine and ribavirin IC_{50}s were 0.4 ± 0.2 and 5.7 ± 2.3, respectively, for A/Texas/1/77/H3N2, and 58 ± 18 and 4.9 ± 1.5, respectively, for B/Rochester/77-SH.

No detailed studies of drug toxicity for MDCK cells were undertaken. Visible cytotoxicity in drug control monolayers was occasionally evident at amantadine or rimantadine concentrations of 100 to 200 μg/ml. In accord with previous observations (16, 24), no cytotoxicity was apparent for ribavirin concentrations of 12.5 μg/ml or lower.

Comparative drug susceptibilities. The cumulative results of drug susceptibility tests for a variety of influenza A and B viruses are listed in Table 1. For viruses with which three or more assays were performed, the results are listed as the mean ± standard deviation. Otherwise, the results of individual assays are recorded. Inosiplex and rifampin are not listed, because no consistent plaque-inhibitory activity was observed at concentrations of less than 100 μg/ml. These drugs also did not show antiviral activity

TABLE 1. *Comparative activity of anti-influenza drugs in a plaque inhibition assay*

Virus strain	IC_{50} (μg/ml)[a]		
	Amantadine	Rimantadine	Ribavirin
A/Victoria/3/75/H3N2	0.4 (0.2)	0.4 (0.3)	5.3 (2.5)
A/Texas/1/77/H3N2	0.4 (0.1)	0.3 (0.2)	5.7 (2.3)
A/USSR/90/77/H1N1	0.2 (0.2)	0.2 (0.1)	6.8 (0.8)
A/Brazil/78/H1N1	NT[b]	0.3	3.2
A/Fukushima/78/H1N1	NT	0.2	3.6
A/New Jersey/8/76/HSW1N1	0.2, 0.2	0.1, 0.2	4.0
A/PR/8/34/H0N1	6.2 (3.4)	7.9, 6.9	2.8, 5.2
B/Rochester/77-SH	102(31)	58(18)	4.9 (1.5)
B/Hong Kong/76	125	86(26)	2.6 (0.8)
B/Leningrad/74	100, 71	45, 59	6.1, 6.3
B/Maryland/1/71	110, 159	82(39)	4.1 (1.3)

[a] Results are expressed as the results of individual assays or as mean ± standard deviation when multiple assays were performed.
[b] NT, Not tested.

at concentrations of 100 μg/ml in other assays involving suppression of infectious virus yield or hemagglutinin production in MDCK monolayers (F. G. Hayden, unpublished data).

For contemporary strains of influenza A viruses, the IC_{50}s of amantadine and rimantadine ranged from 0.1 to 0.4 μg/ml and were consistently 10- to 40-fold lower than those observed with ribavirin. Both amantadine and rimantadine were considerably less active against the highly passaged A/PR/8/34/H0N1 strain, which previous studies have also found to be relatively amantadine resistant (13, 20). In 10 paired experiments involving five different strains of influenza A viruses, the rimantadine IC_{50}s were equal to or slightly lower than those for amantadine ($P < 0.01$; t test). However, the mean IC_{50}s for both drugs were less than or equal to 0.4 μg/ml. Figure 1 shows the inhibition of plaque formation by A/Victoria/3/75/H3N2 and A/Texas/1/77/H3N2 over a range of drug concentrations. Although analysis of covariance demonstrated that rimantadine's effect over this concentration range was greater than that of amantadine ($P < 0.001$), the differences in extent of inhibition were quite small for any one concentration.

Amantadine and rimantadine were over 100-fold less active against representative strains of influenza B viruses. In over 20 paired experiments with influenza B viruses, rimantadine was 1.5 to 2 times more active than amantadine ($P < 0.01$; t test), but the IC_{50}s for both drugs were above 40 μg/ml. These values were 10 to 20-fold higher or more than those for ribavirin. Irrespective of amantadine susceptibility, ribavirin displayed inhibitory activity at concentrations

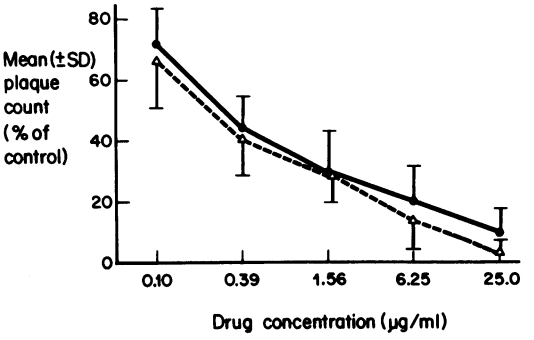

FIG. 1. *Inhibition of plaque formation by A/Victoria/3/75/H3N2 and A/Texas/1/77/H3N2 with increasing concentrations of amantadine hydrochloride (●) and rimantadine hydrochloride (△). Results are expressed as mean (± standard deviation) percentage of plaques compared to infected control monolayers.*

of 2.6 to 6.8 μg/ml for both influenza A and B viruses.

Drug susceptibility of epidemic isolates. Variations in drug susceptibility were determined for multiple isolates of virus obtained during outbreaks of influenza A and B virus infections. The mean (± standard deviation) IC_{50}s for 12 B/Rochester/77 isolates obtained during a 1977 outbreak were 47 ± 11 μg of rimantadine per ml, 104 ± 19 μg of amantadine per ml, and 4.7 ± 1.5 μg of ribavirin per ml. The mean (± standard deviation) IC_{50} for four A/USSR/90/77/H1N1 isolates obtained during an 1978 outbreak was 0.2 ± 0.2 μg of amantadine per ml. The variability of these results is within the reproducibility of the assay method, and the findings do not indicate substantial differences

in drug susceptibility for multiple isolates obtained during the same outbreak.

DISCUSSION

The plaque inhibition assay used in the present study proved to be a rapid, reproducible method for determining drug susceptibility of influenza viruses in vitro. Previous studies have reported a wide range of inhibitory drug concentrations for amantadine, rimantadine, and ribavirin against influenza viruses. These discrepancies are in part due to variations in test methods, which have encompassed a variety of cell cultures, virus strains and inocula, endpoints of infection, and definitions of drug activity. For example, Togo reported that amantadine's minimal inhibitory concentrations for several H3N2 subtype influenza A viruses were 0.05 to 0.1 μg/ml in rhesus monkey kidney (RMK) cell cultures (37), whereas Grunert and Hoffmann reported that amantadine 50% effective doses were 3.1 to 7.5 μg/ml against four H3N2 subtype viruses (12) in RMK monolayers. Tisdale and Bauer found that the ribavirin IC_{50}s differed approximately 10-fold for four influenza A viruses between results in a calf kidney cell plaque assay (35) and those in chicken embryo cell culture (34). Unlike these studies, the present assay utilizes a readily available, continuous cell line, which in conjunction with trypsin allows a wide range of influenza A and B virus to form plaques with high efficiency (36).

More importantly, the plaque inhibition assay used in the present study appeared to be a clinically relevant method of determining drug susceptibility of influenza viruses. The results correlated well with achievable drug levels in humans and with the findings of clinical trials. For example, the present study found that amantadine IC_{50}s ranged from 0.2 to 0.4 μg/ml for contemporary strains of influenza A viruses; these values corresponded well to previously reported results with other plaque assays (2, 20). Peak blood levels of amantadine after a single 2.5-mg/kg or 5.0-mg/kg dose are 0.3 and 0.6 μg/ml, respectively (4). With repeated administration, steady-state blood levels of approximately 0.5 (range 0.1 to 1.2) μg/ml (200 mg per day) and 0.8 to 1.2 (range 0.6 to 1.4) μg/ml (300 mg per day) have been found (1, 11, 27, 31). Amantadine levels in nasal secretions after oral administration appear to be similar to those found in blood (31). The amantadine IC_{50}s found with the plaque inhibition assay fell within achievable levels of amantadine and correctly predicted amantadine's prophylactic and therapeutic activity in human infections (22, 40).

In contrast, the amantadine IC_{50} for the highly passaged A/PR/8/34/H0N1 strain was over 15-fold higher than those found for contemporary influenza A viruses. Grunert et al. reported that experimental murine infection due to this virus strain is resistant to the antiviral action of amantadine (13). Similarly, the amantadine IC_{50}s for influenza B viruses were more than 100-fold greater than those for influenza A viruses and are much higher than achievable blood levels of amantadine. Amantadine has been shown to have no prophylactic (32) or therapeutic (8) effectiveness in infections due to influenza B viruses.

Rimantadine hydrochloride (1-methyl-1-adamantanine methylamine) is a structural analog of amantadine and is similar in its antiviral spectrum. Previous in vitro (35, 39) and animal model (29, 39) investigations have suggested that rimantadine has greater antiviral activity than amantadine against influenza A viruses. In the plaque inhibition assay rimantadine showed marginally greater antiviral activity than amantadine for contemporary strains of influenza A viruses. Double-blind, placebo-controlled trials of the comparative activity of these drugs have documented a significant therapeutic and antiviral effect for each in naturally occurring influenza A virus infections due to H3N2 (40) or H1N1 (VanVoris et al., 18th ICAAC, abstr. no. 483) subtypes, but have not demonstrated superior effectiveness of either.

In the present study ribavirin manifested inhibitory activity for most influenza A and B virus strains in the 3- to 6-μg/ml range and was substantially more active than amantadine or rimantadine for influenza B viruses. These inhibitory concentrations are higher than those reported by Togo (37) but similar to those observed by other workers (3, 24). Single oral doses of 400 mg of ribavirin result in peak serum levels of 0.2 μg/ml between 1 and 2 h after drug ingestion (17). Achievable serum levels are considerably lower than the ribavirin IC_{50}s found in the present study, so that one would not anticipate substantial antiviral activity for ribavirin in human influenza virus infections. Double-blind, controlled studies of ribavirin prophylaxis (600 mg per day) in experimentally induced influenza A (7) and B (38) virus infections have found little evidence of protection and no effect on virus shedding. Although Magnussen et al. (21) have reported a modest but significant effect on illness severity and virus shedding with larger doses of ribavirin (1.0 g per day) begun 6 h after A/Victoria/3/75/H3N2 infection of volunteers, the same ribavirin dose was not associated with therapeutic or antiviral activity in natural influenza A virus infection due to an H1N1 subtype (C. B. Smith, personal communication).

Inosiplex at concentrations of 100 μg/ml or

lower manifested no reproducible antiviral activity in the present study. Previous in vitro and animal model studies of inosiplex's antiviral activity have yielded conflicting results (5, 10, 23). The purine portion of the drug is apparently metabolized in certain cell culture systems (T. Ginsberg, R. Settineri, E. Padenhauer, and A. J. Glasky, Abstr. Annu. Meet. Am. Soc. Microbiol., 1973, V73, p. 206), so that drug activity might not be present after several hours in a plaque assay system. In experimentally produced A/Hong Kong/8/68/H3N2, Longley et al. found that inosiplex (5.0 g per day) did not protect against illness but did reduce virus isolation rates slightly as compared to placebo (19). Clinical trials of inosiplex's therapeutic effect in naturally acquired influenza have not been reported, but the results of the present study would predict a lack of substantial antiviral activity. In vitro drug susceptibility testing would of course fail to detect immunomodulating effects, which have been attributed to this drug (9, 14).

Previous studies have shown that there is considerable strain variability in amantadine susceptibility of influenza A viruses (28, 34). In the present study there was little variation in amantadine activity against representative strains of contemporary influenza A or B viruses or against multiple isolates obtained during distinct epidemic periods. These discrepancies may in part be secondary to differences in passage history, since amantadine susceptibility has been reported to decrease with increasing passage level in animals (34). The highly passaged H0N1 subtype virus, which has previously been reported to be relatively amantadine resistant (2, 13, 20), was also found to have a considerably higher amantadine IC_{50} than the other influenza A viruses tested. However, in contrast to the findings of Grunert and Hoffman (12), the A/New Jersey/8/76/HSW1N1 strain was not found to be dramatically more susceptible to amantadine than other contemporary influenza A viruses.

Amantadine-resistant influenza A virus can be selected by passage of virus in cell culture, embryonated eggs, and mice in the presence of amantadine (6, 25, 26). Oxford and Potter found that the inhibitory concentration of amantadine increased from 0.1 μg/ml to 5 to 12.5 μg/ml for an A2/Singapore/1/57 strain serially passaged in mice receiving high amantadine doses (26). Amantadine resistance is a stable genetic characteristic that relates to the gene coding for M protein (20) and can be transfered to amantadine-susceptible virus (2). Attempts to recover amantadine-resistant variants from persons receiving amantadine orally (26) or by inhalation (F. G. Hayden, W. J. Hall, R. G. Douglas, Jr., and D. M. Speers, 18th ICAAC, abstr. no. 485) have been unsuccessful to the present, but drug resistance remains an important clinical consideration. The present assay method can detect this level of drug resistance and could be used to screen for amantadine-resistant virus in persons receiving this drug.

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