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Phenotypic Drug Susceptibility Assay for Influenza Virus Neuraminidase Inhibitors

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A flow cytometric (fluorescence-activated cell sorter [FACS]) assay was developed for analysis of the drug susceptibilities of wild-type and drug-resistant influenza A and B virus laboratory strains and clinical isolates for the neuraminidase (NA) inhibitors oseltamivir carboxylate, zanamivir, and peramivir. The drug susceptibilities of wild-type influenza viruses and those with mutations in the hemagglutinin (HA) and/or NA genes rendering them resistant to one or more of the NA inhibitors were easily determined with the FACS assay. The drug concentrations that reduced the number of virus-infected cells or the number of PFU by 50% as determined by the FACS assay were similar to those obtained with the more time-consuming and labor-intensive virus yield reduction assay. The NA inhibition (NAI) assay confirmed the resistance patterns demonstrated by the FACS and virus yield assays for drug-resistant influenza viruses with mutations in the NA gene. However, only the FACS and virus yield assays detected NA inhibitor-resistant influenza viruses with mutations in the HA gene but not in the NA gene. The FACS assay is more rapid and less labor-intensive than the virus yield assay and just as quantitative. The FACS assay determines the drug susceptibilities of influenza viruses with mutations in either the HA or NA genes, making the assay more broadly useful than the NAI assay for measuring the in vitro susceptibilities of influenza viruses for NA inhibitors. However, since only viruses with mutations in the NA gene that lead to resistance to the NA inhibitors correlate with clinical resistance, this in vitro assay should not be used in the clinical setting to determine resistance to NA inhibitors. The assay may be useful for determining the in vivo susceptibilities of other compounds effective against influenza A and B viruses.

RNA viruses, such as influenza virus, have a high rate of mutation. Some of these mutations lead to viruses that are resistant to the currently used antiviral drugs and can be selected in the presence of antiviral drugs. If the drug-resistant viruses are biofit, their replication can lead to serious disease that cannot be treated effectively with the previously used antiviral compounds. This scenario has occurred frequently. When amantadine hydrochloride was used to treat influenza virus type A infections, 30% of the virus isolates obtained from treated patients were found to be resistant (9, 11, 22). With the licensing of the neuraminidase (NA) inhibitors, the selection of influenza viruses resistant to these inhibitors was of concern (32, 39, 43, 52, 61). In vitro resistance associated with amino acid substitutions in the hemagglutinin (HA) or NA antigens or both has been reported for the NA inhibitors (4, 14, 15, 32, 40, 49, 55). Despite these concerns, recent reports have demonstrated that there is little or no natural resistance to oseltamivir or zanamivir (5, 33). To determine if mutations to zanamavir occurred in vivo, the drug susceptibilities of clinical isolates obtained during a phase II clinical trial of zanamivir were determined by the plaque reduction assay (PRA), the NA inhibition (NAI) assay, and an in vivo assay using ferrets (3, 17). A comparison of 41 paired isolates obtained before and during therapy with zanamivir showed no shifts in susceptibility to zanamivir when measured by the NAI assay, but the PRA using MDCK cells showed variable susceptibility to zanamivir. The susceptibilities of the clinical isolates determined by the PRA did not correlate with in vivo susceptibility studies in humans and ferrets, whereas the NAI assay did correlate with the in vivo susceptibility assays. In a study of 54 isolates obtained after treatment with oseltamivir, 2 clinical isolates were resistant in the NAI assay and an additional 8 were resistant in the PRA (16). These discrepancies between the PRA and the NAI assay could be due to the isolation of viruses with mutations in the HA gene that lead to in vitro resistance. NA inhibitor-resistant viruses with mutations in the HA gene would be scored in the PRA, but not in the NAI assay. The close relationship between the drug susceptibilities obtained with the NAI assay and the in vivo assays suggests that for these clinical isolates the NAI assay correlates better with the in vivo assay than the PRA for the NA inhibitors. The present evidence suggests that only mutations in the NA gene that lead to resistance to the NA inhibitors are clinically relevant.

The currently used in vitro drug susceptibility assays, such as the PRA, the virus yield reduction assay, and the neutral red dye uptake assay, are cumbersome, time-consuming, and subjective (21, 45). A PCR-based drug susceptibility assay has recently been published, but its usefulness in clinical trials has not been evaluated (54). Previously, we demonstrated that the susceptibilities of herpes simplex viruses and human cytomegalovirus for antiviral compounds could be determined by flow

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TABLE 1. Description of influenza virus laboratory strains and clinical isolates used in these studies

Virus	Type	Phenotype	Mutation	Reference or source
PR/8/34 (H1N1)	A	WT[d]	None	P. Wagaman
PR/8/34 (H1N1) R_2A[a,e]	A	Resistant to peramivir	HA R208K; N129S No NA mutations	
Shangdong/09/93 (H3N2)	A	WT	None	49
Shangdong/09/93 (H3N2)R_2A[e]	A	Resistant to peramivir	HA K189E No NA mutations	
Texas/36/91 (H1N1)[b]	A	WT	None	16
Texas/36/91 (H1N1) 35.9[b]	A	Resistant to oseltamivir	HA T137A, T225G NA H274Y	
Yamagata	B	WT	None	4
Yamagata R_2A[e]	B	Resistant to peramivir	6 HA mutations NA H273Y	
Memphis/20/96[b,c]	B	WT	None	17
Memphis/20/96 Lys152[b]	B	Resistant to zanamivir	HA T198I NA R152K	
Memphis/20/96 Ile198[c]	B	Resistant to zanamivr	HA T198I	17
23 clinical isolates	A	WT	None known	
12 clinical isolates	B	WT	None known	

[a] The PR/8 virus mutant that was grown in the presence of increasing concentrations of peramivir contained two mutations in the HA gene and none in the NA gene (P. Wagaman, unpublished data).
[b] Wild-type and zanamivir- or oseltamivir-resistant strains of influenza virus were obtained from Larisa V. Gubareva, University of Virginia, Charlottesville, Va.
[c] Wild-type and zanamivir-resistant mutant strains of influenza virus obtained from Robert G. Webster, St. Jude Children's Research Hospital, Memphis, Tenn.
[d] WT = wild type.
[e] R_2A = peramivir-resistant virus.

cytometric (fluorescence-activated cell sorter [FACS]) analysis of virus-infected cells treated with virus antigen-specific fluorochrome-labeled monoclonal antibodies (34). The FACS assay is rapid and quantitative. For herpes simplex viruses and human cytomegalovirus, the EC_{50} values (the concentrations of drug that reduce the number of virus-infected cells by 50%) obtained by FACS analysis correlate with those obtained with the virus yield reduction assay and the PRA (8, 35–37). In this proof-of-principle report, we show that fluorochrome-labeled monoclonal antibodies to influenza virus type A or type B nucleocapsid antigens and FACS analysis can be used to determine the in vitro drug susceptibilities of influenza virus laboratory strains and clinical isolates to the NA inhibitors peramivir, zanamivir, oseltamivir carboxylate, and ribavirin.

MATERIALS AND METHODS

Cells and viruses. MDCK cells (ATCC CCL-34) were obtained from the American Type Culture Collection and maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% MEM nonessential amino acids, 1% penicillin-streptomycin solution, and 1% glutamine (Invitrogen). MDCK cells were grown as monolayers in 75-cm^2 or 25-cm^2 cell culture flasks or 60-mm petri dishes (Corning). Passages 62 to 75 were used for these experiments

Table 1 lists the properties and sources of wild-type and NA inhibitor-resistant laboratory strains and clinical isolates of influenza viruses. Influenza virus clinical isolates, collected between 1995 and 2000, were obtained from the Clinical Microbiology Laboratory at the Albany Medical Center Hospital, the New York State Health Department, and the Provincial Laboratory, Regina, Saskatchewan, Canada. Use of clinical isolates for study was approved by the Albany Medical College Institutional Review Board. Stocks of wild-type viruses were prepared by diluting virus in MEM supplemented with 0.2% bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, Mo.) and 2 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Sigma Chemical Company)/ml (virus growth medium) and infecting MDCK cell monolayers at a multiplicity of infection (MOI) of 0.001 PFU per cell. After a 1-h adsorption period at 37°C, the inoculum was removed, 5 ml of virus growth medium was added to each flask, and the flasks were then incubated at 35°C until cytopathic effect (cell rounding) was evident throughout the monolayer. The medium containing released virus, virus-infected cells, and some debris was collected and centrifuged at 800 × *g* for 10 min. The clarified supernatants containing cell-free virus were stored at −70°C. Titers of virus stocks were determined by plaque assay on MDCK cell monolayers. Stocks of drug-resistant viruses were prepared by growing viruses in the presence of virus growth medium supplemented with antiviral compound (1 or 10 μM) and processed as described above for wild-type viruses. The uncharacterized clinical isolates obtained from the clinical microbiology laboratories used in this study were passed only twice in MDCK cells before determining the EC_{50} values by FACS or virus yield reduction assays and the 50% inhibitory concentration (IC_{50}) values by the NAI assay.

Antiviral drugs. Ribavirin was purchased from Sigma Chemical Company. Peramivir, zanamivir, and oseltamivir carboxylate were provided by R. W. Johnson Pharmaceutical Research Institute, Raritan, N.J. The drugs were prepared in sterile glass-distilled water to a final concentration of 1 mg/ml and used immediately.

FACS drug susceptibility assay. The effect of antiviral compounds on the replication of influenza viruses in MDCK cells was determined by flow cytometric analysis of virus-infected cells. The procedure was essentially as described previously with the following modifications (8, 34–37). Briefly, 2-day-old confluent MDCK cell monolayers in 25-cm^2 flasks were washed twice with MEM supplemented with 0.2% BSA and infected with influenza virus at an MOI of 0.001 PFU per cell in 1 ml of virus growth medium supplemented with various concentrations of antiviral compounds. After a 1-h adsorption period at 37°C, the inoculum was removed and 5 ml of virus growth medium supplemented with various concentrations of an antiviral compound was added to each flask. After 16 h of incubation at 35°C, the medium containing released virus and some floating cells was collected, cell debris was removed by centrifugation at 800 × *g* for 10 min, and the clarified supernatants containing released virus were stored at −70°C for analysis in the virus yield reduction assay. The virus-infected cells that remained attached to the flask were harvested with 0.25% trypsin–0.03% EDTA, permeabilized with methanol, and treated with a fluorochrome-labeled monoclonal antibody to a type-specific influenza virus nucleocapsid antigen (influenza A and B DFA kit; catalog no. 3123; Chemicon International, Inc., Temecula, Calif.). The number of antigen-positive cells at each drug concentration was determined by FACS analysis. Initially, intact cells were identified by FACS analysis of their forward and right angle light scatter properties. Ten thousand events with the appropriate light scatter properties were collected within a gate and then analyzed for fluorescence intensity. Uninfected and influenza A or B virus-infected MDCK cells treated with the appropriate fluorochrome-labeled monoclonal antibody served as negative and positive controls, respectively. The fluorescence intensities of the cells in the uninfected cell populations were within the first decade on the fluorescence intensity scale. Using

TABLE 2. Average EC_{50} values for NA inhibitors for peramivir-susceptible and -resistant influenza viruses: FACS assay

Strain	Avg EC_{50} (μM)[a]			
	Peramivir	Oseltamivir	Zanamivir	Ribavirin
A/PR/8/34 WT[b]	0.002 ± 0.001	0.003 ± 0.001	0.063 ± 0.048	3.03 ± 1.30
A/PR/8/34 R_2A[c]	>20 (>10,000)[d]	>20 (>6,666)	>20 (>317)	6.82 ± 3.59
A/Shangdong WT	0.001 ± 0.001	0.002 ± 0.001	0.011 ± 0.015	8.21 ± 4.91
A/Shangdong R_2A	>20 (>10,000)	>20 (>10,000)	>20 (>1,818)	10.19 ± 5.30
B/Yamagata WT	0.022 ± 0.005	0.229 ± 0.001	0.129 ± 0.001	2.59 ± 1.64
B/Yamagata R_2A	>20 (>909)	3.520 ± 0.01 (15)	0.574 ± 0.036 (4)	3.03 ± 0.87

[a] EC_{50} values are the average of at least three determinations for each virus-antiviral combination. Values are means ± standard deviations.
[b] WT = wild type.
[c] R_2A = resistant viruses selected against peramivir.
[d] The number in parentheses is the number of times that the average EC_{50} value of the resistant strain was greater than the average EC_{50} value of the wild-type strain.

the gate that was set for the uninfected cells, the fluorescence intensities of virus-infected cells fell at least one decade beyond the first decade on the fluorescence intensity scale. The Cyteron absolute flow cytometer calculated the percentage of antigen-positive cells.

Virus yield reduction assay. To determine the effect of the NA inhibitors on the yield of infectious virus, the number of PFU present in the clarified medium at each drug concentration was determined by plaque assay as described by Sidwell and Smee (45). Serial 10-fold dilutions of clarified virus supernatant were made in virus growth medium. The MDCK cell monolayers were washed twice with MEM supplemented with 0.2% BSA, and 0.5 ml of each virus dilution was added to the monolayers in triplicate. After a 1-h adsorption period at 37°C under an atmosphere of 5% CO_2, the inoculum was removed and 5 ml of virus growth medium supplemented with 1% DEAE dextran and 0.5% agar was added to each plate. After 2 days of incubation at 37°C under an atmosphere of 5% CO_2, the agar was removed, the monolayers were stained with 0.1% crystal violet in 20% ethanol, and the number of PFU were counted by hand.

NAI assay. The procedure for the NAI assay was that described by Gubareva et al. (18). The NA activity of each virus sample was determined by a fluorometric assay that measured 4-methylumbelliferone released from the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid by the enzymatic activity of the influenza virus NA (42). Virus dilutions containing between 400 and 800 fluorescence units were used in the NAI assay. Twofold dilutions of virus were incubated with various concentrations of the NA inhibitors for 1 h followed by addition of NaOH to stop the reaction. The fluorescence was measured using a fluorometer with an excitation wavelength of 365 nm and an emission wavelength of 460 nm. The concentration of compound that reduced the fluorescence intensity by 50% was the IC_{50} value.

Statistical analysis. The percent reductions in the number of antigen-positive cells in the FACS assay, the number of PFU in the virus yield reduction assay, and the mean fluorescence intensity in the NAI assay at each compound concentration were calculated. EC_{50} values (the concentration of compound that reduced the number of antigen-positive cells in the FACS assay or the number of PFU in the virus yield assay by 50%) or IC_{50} values (the concentration of compound that reduced the mean fluorescence intensity in the NAI assay by 50%) were determined by plotting the percent reduction against the concentration of compound using the SlideWrite Plus software (Advanced Graphics Software, Encinitas, Calif.). Each value is the average of at least three separate determinations.

RESULTS

Determination of EC_{50} values of wild-type and peramivir-resistant laboratory strains of influenza viruses by the FACS assay. Researchers at the R. W. Johnson Pharmaceutical Research Institute produced influenza virus laboratory strains resistant to the NA inhibitor peramivir by passing wild-type viruses in the presence of increasing concentrations of the compound (4, 49; P. Wagaman, personal communication). The drug susceptibilities of wild-type and peramivir-resistant (R_2A) influenza virus laboratory strains to various NA inhibitors and ribavirin were determined by the FACS assay (Table 2). The average EC_{50} values for wild-type PR/8 and wild-type Shangdong influenza A viruses for peramivir and oseltamivir were between 1 and 3 nM and those for wild-type PR/8 and Shangdong influenza A viruses for zanamivir were 20- and 10-fold higher, respectively. The EC_{50} values for the peramivir-resistant PR/8 and peramivir-resistant Shangdong influenza A viruses were greater than 20 μM for all three of the NA inhibitors and 317 to greater than 10,000 times the EC_{50} values of the wild-type influenza A viruses. The average EC_{50} values for the wild-type Yamagata influenza B virus for the three NA inhibitors were approximately 10 to 100 times higher than those for the influenza A viruses. This difference in the EC_{50} values of NA inhibitors between influenza A and B viruses has been reported by others using more traditional phenotypic assays (1, 2, 5, 48, 59). The EC_{50} values of the peramivir-resistant Yamagata influenza B virus were 909 times greater for peramivir, 15 times greater for oseltamivir, and only 4 times greater for zanamivir than the EC_{50} values for the wild-type B/Yamagata virus. The average EC_{50} values for the NA inhibitors for the permavir-resistant Yamagata influenza B virus were more variable than those seen with the permavir-resistant influenza A viruses. As expected, there was no difference in the average EC_{50} value between the wild-type and resistant influenza type A and B viruses for ribavirin. These results showed that the FACS assay can be used to determine the EC_{50} values of influenza viruses for these NA inhibitors and that it can be used to distinguish between susceptible and resistant influenza virus laboratory strains for these antiviral compounds. The data show that there is uniform qualitative, but not quantitative, cross-resistance between the three NA inhibitors for these peramivir-resistant influenza A viruses but variable cross-resistance for the peramivir-resistant influenza B virus.

Effect of NA inhibitors on virus yield in MDCK cells infected with wild-type or peramivir-resistant influenza viruses. The data presented above show that EC_{50} values for NA inhibitors can be determined by FACS assay. This is the first demonstration of the use of a FACS assay to determine the drug susceptibilities of influenza A and B viruses to antiviral compounds. To confirm these results, virus yield reduction assays were performed on the clarified supernatants removed from the monolayers used in the FACS assay (Table 3). The average EC_{50} values for all three wild-type influenza viruses were similar to those determined by the FACS assay (Table 2). The average EC_{50} values for these NA inhibitors for wild-type B/Yamagata influenza virus were higher than those for the influenza A viruses, a finding similar to those obtained with the

TABLE 3. Average EC_{50} values for NA inhibitors for peramivir-susceptible and resistant influenza viruses: virus yield assay

Strain	Avg EC_{50} (μM)[a]		
	Peramivir	Oseltamivir	Zanamivir
A/PR/8/34 WT[b]	0.002 ± 0.002	0.008 ± 0.001	0.002 ± 0.001
A/PR/8/34 R_2A[c]	>2.0 (>1,000)[d]	>2.0 (>250)	0.24 (120)
A/Shangdong WT	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001
A/Shangdong R_2A	>2 (>2,000)	>2 (>2,000)	0.197 (197)
B/Yamagata WT	0.087 ± 0.011	0.615 ± 0.007	0.022 ± 0.01
B/Yamagata R_2A	>2.0 (>23)	>2.0 (>3)	0.02 (1)

[a] EC_{50} values are the average of at least three determinations for each virus-antiviral combination. Values are means ± standard deviations.
[b] WT = wild-type viruses.
[c] R_2A = resistant viruses selected against peramivir.
[d] The number in parentheses is the number of times that the average EC_{50} value of the resistant strain was greater than the average EC_{50} value of the wild-type strain.

FACS assay (Table 2). Both assays clearly distinguished between wild-type and the NA inhibitor-resistant viruses. However, the magnitudes of the average EC_{50} values between wild-type and NA inhibitor-resistant virus pairs were not as great as those demonstrated in the FACS assay. Nevertheless, this comparison helps to validate the FACS assay as a useful in vitro method for determining the drug susceptibilities of these influenza virus laboratory strains to the NA inhibitors.

Effect of NA inhibitors on the NA activity of wild-type and peramivir-resistant influenza virus laboratory strains. The FACS assay is a novel way to determine the susceptibilities of influenza viruses to NA inhibitors. Many investigators rely on the NAI assay to determine the susceptibilities of influenza virus clinical isolates to the NA inhibitors (18, 19, 52, 57). To test if the results of the FACS assay correlated with those of the NAI assay, the drug susceptibilities of wild-type and peramivir-resistant influenza A and B virus laboratory strains to the three NA inhibitors were determined by the NAI assay (Table 4). There were essentially no differences between the average IC_{50} values for the NAI assay for the wild-type and peramivir-resistant PR8 and Shangdong influenza A viruses to peramivir, oseltamivir, and zanamivir. These results reflect the fact that there were no mutations in the NA genes of these two influenza A viruses that lead to resistance to these NA inhibitors (Table 1). In contrast, there was a 156-fold difference in the IC_{50} value for peramivir between the wild-type and peramivir-resistant influenza B viruses. This large difference was due to a mutation in the NA gene that converted His273 to Tyr273 in the NA of the influenza B virus, which contributes to resistance to peramivir. This large difference in the IC_{50} values for the NAI assay was not reflected in the NA activity of the influenza B viruses for oseltamivir and zanamivir, which showed only a sixfold difference and no difference, respectively. Similar results were recently reported by Baum et al. (4). These results showed that there is little cross-resistance contributed by the NA between the three NA inhibitors for this influenza B virus laboratory strain. In contrast, the FACS assay and the virus yield reduction assay showed cross-resistance for these NA inhibitors for both influenza A viruses but not for the influenza B virus (Table 2). These cross-resistances must be due to mutations in the HA gene of the two influenza A viruses, since there are no known mutations in the NA genes of the influenza A viruses used in these experiments.

FACS drug susceptibility assay for NA inhibitors for susceptible and resistant influenza A and B virus clinical isolates. The previous data showed that the FACS assay can distinguish between drug-susceptible and drug-resistant laboratory strains of influenza A and B viruses. To determine if the FACS assay can also be used to study drug susceptibilities of influenza A and B virus clinical isolates, we obtained three pairs of influenza virus clinical isolates from either Robert Webster or Larisa V. Gubareva (Table 1). Stocks of these viruses were prepared by growing the wild-type viruses in the absence of compound and growing the drug-resistant viruses in the presence of a 1 or 10 μM concentration of the compound. Each virus pair was grown in the presence of compound and analyzed for the effect of drug on the number of virus-infected cells by FACS, the number of released viruses by the virus yield reduction assay, and the NA activity associated with each virus by the NAI assay (Table 5). In all three phenotypic assays, the wild-type A/Texas/36/91 and B/Memphis/20/96 influenza viruses were sensitive to the NA inhibitors, with EC_{50} values for the wild-type influenza B viruses approximately 10-fold higher than those for the influenza A viruses. The mutant influenza virus A/Texas/36/91 (HA, T225G; NA, H274Y), selected against oseltamivir, was very resistant to peramivir and oseltamivir, but less resistant to zanamivir in all three assays. The single mutant B/Memphis/20/96 (HA, T198I), obtained after growth in the presence of 10 μM peramivir, showed resistance to peramivir in the FACS and virus yield reduction assays but not in the NAI assay. This result is expected, because this mutant has no mutations in the NA gene that lead to resistance to the NA inhibitors (Table 1). According to the FACS and

TABLE 4. Average IC_{50} values for NA inhibitors for wild-type and peramivir resistant influenza virus laboratory strains: NAI assay

Strain	Avg IC_{50} (nM)[a]		
	Peramivir	Oseltamivir	Zanamivir
A/PR8/34 WT[b]	0.045 ± 0.009	0.559 ± 0.150	0.126 ± 0.018
A/PR8/34 R_2A[c]	0.082 ± 0.029	0.766 ± 0.104	0.109 ± 0.054
A/Shangdong WT	0.065 ± 0.038	0.035 ± 0.001	0.287 ± 0.065
A/Shangdong R_2A	0.071 ± 0.024	0.042 ± 0.008	0.285 ± 0.057
B/Yamagata WT	0.215 ± 0.010	4.983 ± 2.882	1.292 ± 0.317
B/Yamagata R_2A	33.585 ± 9.84 (156)[d]	29.439 ± 7.20 (6)	0.553 ± 0.172 (0.42)

[a] IC_{50} values are the average of at least three independent observations. Values are means ± standard deviations.
[b] WT = wild-type viruses.
[c] R_2A = resistant viruses selected against peramivir; phenotype is based on the PRA results.
[d] The number in parentheses is the number of times that the average IC_{50} value of the resistant strain is greater than the average IC_{50} value of the wild-type strains.

TABLE 5. Comparison of phenotypic assays for NA inhibitors for wild-type and mutant influenza virus clinical isolates[a]

Strain and inhibitor	FACS assay result (μM)	Yield assay result (μM)	NAI assay result (nM)
A/Texas/36/91 wild type			
PERAMIVIR	0.002 ± 0.001	0.001 ± 0	0.056 ± 0.011
OSELTAMIVIR	0.002 ± 0.001	0.002 ± 0	0.220 ± 0.037
ZANAMIVIR	0.008 ± 0.009	0.001 ± 0	0.125 ± 0.043
A/Texas/36/91 (HA:T225G; NA:H274Y) selected against oseltamivir			
PERAMIVIR	1.996 ± 0.656 (998)[b]	0.056 ± 0.046 (56)	44.572 ± 8.596 (990)
OSELTAMIVIR	1.720 ± 0.670 (860)	2.234 ± 0.896 (1,117)	>1,000 (>4,545)
ZANAMIVIR	0.018 ± 0.002 (2.23)	0.012 ± 0.008 (12)	4.146 ± 1.296 (33)
B/Memphis/20/96 wild type			
PERAMIVIR	0.035 ± 0.023	0.081 ± 0.005	0.094 ± 0.016
OSELTAMIVIR	0.104 ± 0.082	0.021 ± 0.001	6.575 ± 2.838
ZANAMIVIR	0.079 ± 0.067	0.003 ± 0.002	0.742 ± 0.414
B/Memphis/20/96 (HA:T198I) selected against peramivir			
PREAMIVIR	0.122 ± 0.019 (68)	0.101 ± 0.115 (24)	0.130 ± 0.022 (0.89)
OSELTAMIVIR	0.683 ± 0.123 (5)	0.142 ± 0.076 (9)	5.512 ± 3.414 (1.13)
ZANAMIVIR	0.082 ± 0.012 (0.4)	0.002 ± 0.0002 (0.3)	0.704 ± 0.359 (1.17)
B/Memphis/20/96 wild type			
PERAMIVIR	0.069 ± 0.056	0.008 ± 0.004	0.129 ± 0.01
OSELTAMIVIR	0.183 ± 0.032	0.118 ± 0.011	3.420 ± 0.819
ZANAMIVIR	0.059 ± 0.01	0.002 ± 0.001	0.515 ± 0.083
B/Memphis/20/96 (HA:T198I; NA:R152K) selected against zanamivir			
PERAMIVIR	2.793 ± 0.112 (40)	0.273 ± 0.012 (34)	81.448 ± 15.308 (631)
OSELTAMIVIR	1.720 ± 0.011 (9.39)	0.943 ± 0.01 (8)	320.259 ± 197.842 (94)
ZANAMIVIR	>20 (>340)	>2.0 (>1,000)	36.871 ± 21.710 (71.59)

[a] EC_{50} and IC_{50} values are the average of at least three determinations for each assay. Values are means ± standard deviations.
[b] The number in parentheses is the number of times that the average EC_{50} or IC_{50} value of the resistant strain was greater than the average EC_{50} or IC_{50} value of the wild-type strain.

virus yield reduction assays, this single mutant was less resistant to oseltamivir than to peramivir, and it was not resistant to zanamivir. The resistance to peramivir must be associated with mutations in the HA gene, since this mutant does not have any mutations in the NA gene that would lead to resistance to the NA inhibitors. The double mutant B/Memphis/20/96 (HA, T198I; NA, R152K), selected against zanamivir, was resistant to peramivir, oseltamivir, and zanamivir by all three assays. It was less resistant to oseltamivir in the FACS and virus yield reduction assays but resistant in the NAI assay. These results suggest that in general, the FACS drug susceptibility assay can distinguish between drug-susceptible and drug-resistant clinical isolates.

Comparison of FACS assay EC_{50} and NAI assay IC_{50} values for ribavirin and NA inhibitors for influenza virus type A and B clinical isolates. The above data showed that the FACS assay can be used to distinguish between drug-susceptible and drug-resistant laboratory strains and clinical isolates selected in the presence of NA inhibitors. The FACS assay was used to determine the EC_{50} values of fresh influenza A and B virus clinical isolates to NA inhibitors (Table 6). Average EC_{50} values for 23 type A influenza virus clinical isolates for peramivir, oseltamivir, and zanamivir were similar to those reported for the wild-type laboratory strains (Table 2). The average EC_{50} values for 12 type B influenza virus clinical isolates were 64 times higher for peramivir, 8 times higher for oseltamivir, and less than 2 times higher for zanamivir than the EC_{50} values for these compounds for influenza A viruses. This difference in the average EC_{50} values for NA inhibitors between influenza A and B viruses was also shown above for the laboratory strains (Table 2) and by others using different phenotypic assays (1, 2, 5, 48, 59). The NAI assay showed that these 23 type A and 12 type B influenza virus natural clinical isolates were sensitive to all three NA inhibitors. Ribavirin was not tested in the NAI assay because it should have no effect on the activity of the NA. These results showed that there is little or no resistance to the NA inhibitors in unselected influenza A and B virus clinical

TABLE 6. Comparison of assays for antiviral drugs for influenza virus clinical isolates

Drug	Assay result[a]			
	FACS		NAI	
	A	B	A	B
Peramivir	0.003 ± 0.001	0.194 ± 0.002	0.218 ± 0.1	0.194 ± 0.102
Oseltamivir	0.004 ± 0.001	0.033 ± 0.009	0.137 ± 0.038	4.026 ± 0.870
Zanamivir	0.018 ± 0.013	0.028 ± 0.006	0.586 ± 0.116	0.628 ± 0.039
Ribavarin	11.00 ± 6.35	9.26 ± 4.15	NA	NA

[a] Values are averages (23 influenza virus type A isolates and 12 influenza virus type B isolates were tested). For the FACS assay, EC_{50}s (micromolar concentrations) are given, and for the NAI assay, IC_{50}s (nanomolar concentrations) are given. NA, not applicable.

isolates. These findings confirmed recent reports that used the NAI assay and sequence analysis of the NA gene to show that there was little or no natural resistance to zanamivir or oseltamivir in the influenza A and B virus populations isolated between 1999 and 2002 (5, 33).

DISCUSSION

We have demonstrated that a FACS assay can be used to determine the susceptibilities of wild-type and drug-resistant influenza A and B virus laboratory strains and low-passage clinical isolates for ribavirin and the NA inhibitors peramivir, oseltamivir, and zanamivir. The FACS and virus yield reduction assays gave similar EC_{50} values for the NA inhibitors for wild-type influenza A and B viruses. Both assays clearly distinguished between peramivir-susceptible and -resistant influenza virus type A and type B laboratory strains. The FACS assay and the NAI assay distinguished between a peramivir-susceptible and a peramivir-resistant influenza B virus laboratory strain with a mutation in the NA gene. However, only the FACS and virus yield assays, and not the NAI assay, detected resistance in the two peramivir-resistant influenza A viruses with mutations only in the HA genes. Similar results were obtained when the EC_{50} and IC_{50} values of three pairs of drug-susceptible and drug-resistant influenza virus clinical isolates were determined using the FACS, virus yield reduction, and NAI assays. If the clinical isolate had mutations in both the HA and NA genes that led to drug resistance, then all three assays were able to distinguish between wild-type and drug-resistant clinical isolates. If the drug resistance mutation was only in the HA gene, then only the FACS and virus yield assays could distinguish between wild-type and drug-resistant clinical isolates. The FACS EC_{50} values for wild-type and peramivir-resistant A/Shangdong and B/Yamagata laboratory strains of influenza viruses and fresh influenza A and B clinical isolates for these NA inhibitors were similar to EC_{50} values reported by others using more standard techniques (2, 4, 5, 48, 49). These results show that the FACS assay could be used to determine the drug susceptibilities of influenza A and B viruses for these NA inhibitors.

Every year influenza viruses cause morbidity and mortality among the very young and the elderly. Periodically, pandemics occur with much greater sickness and death. This is the natural course of influenza virus infections in the United States (7). In 1997, an unusual event occurred in the Hong Kong poultry markets. Avian influenza viruses spread directly from infected chickens to people, leading to overt disease in 18 infected individuals with six deaths (50, 60). Extensive investigations on the influenza viruses obtained from individuals who died from these infections have identified some of the genetic changes that occurred in the original avian influenza virus that led to the more deadly virus (20, 44). With this information, one could create influenza viruses like these or that may be considerably more dangerous than this deadly virus and use them as bioweapons. This scenario was recently suggested (28). With the ease with which influenza viruses spread through an unvaccinated population, such a deadly virus could pose a very serious threat. If an influenza virus epidemic or bioterrorist attack occurred, there are a number of antiviral compounds approved by the U.S. Food and Drug Administration for the prophylaxis and treatment of infections due to influenza viruses. These compounds include the ion channel blockers amantadine and rimantadine and the NA inhibitors zanamivir and oseltamivir carboxylate (6, 9, 18, 23, 31, 53, 58). Although effective in the prevention and therapy of influenza, these drugs have several drawbacks. Amantadine and rimantadine are only useful for the treatment of infections caused by type A influenza viruses, drug resistance develops rapidly during therapy, and they have several adverse side effects (9, 11, 22). Zanamivir and oseltamivir carboxylate must be taken within 48 h of symptoms to be effective, a time frame that is not convenient for therapy in the general population (23, 53, 58). These factors limit the use of these antiviral drugs for the prevention and treatment of influenza. The search for better and more useful NA inhibitors is under way. The pyrrolidine-based compound A-315675, the pyrazinecarboximide-based compound T-750, and the cyclopentane peramivir (RWJ-270201) are under study for their activities against influenza type A and B viruses (1, 12, 26, 27, 38). Peramivir has in vitro and in vivo activities equal to or greater than those of zanamivir and oseltamivir carboxylate against a number of influenza type A and B viruses (2, 4, 5, 10, 18, 46–49). The compound is bioavailable after oral treatment and can be given once a day (1, 10, 18, 51). In addition to the NA inhibitors, cyanovir-N, an inhibitor of the influenza virus HA, is also under development (41). The rapid, quantitative FACS assay should be helpful in screening compounds for activity against influenza A and B viruses.

The emergence of influenza viruses resistant to the NA inhibitors appears to be low, but these antiviral drugs have not been widely used and so the true picture of resistance is unknown at this time. Although influenza viruses that are resistant to the NA inhibitors are less pathogenic than wild-type influenza viruses, future influenza epidemics or potential bioterrorist attacks may involve influenza viruses that are both drug resistant and more pathogenic than those present at this time (7, 24, 25). Wild-type influenza viruses found in nature, including the viruses isolated from patients who died in the 1997 Hong Kong epidemic and the avian viruses that circulate throughout the world, are susceptible to the NA inhibitors (13, 29, 30, 56). The susceptibilities of any naturally occurring or weaponized influenza viruses to these antiviral drugs must be known before these drugs can be used effectively. The FACS assay would be very useful for rapidly determining the susceptibilities of any influenza virus that occurred naturally or was produced for the purpose of bioterrorism.

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