JOURNAL OF VIROLOGY, May 1997, p. 3385–3390 Vol. 71, No. 5
0022-538X/97/$04.00+0
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Catalytic and Framework Mutations in the Neuraminidase Active Site of Influenza Viruses That Are Resistant to 4-Guanidino-Neu5Ac2en

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Received 22 November 1996/Accepted 3 February 1997

Here we report the isolation of influenza virus A/turkey/Minnesota/833/80 (H4N2) with a mutation at the catalytic residue of the neuraminidase (NA) active site, rendering it resistant to the novel NA inhibitor 4-guanidino-Neu5Ac2en (GG167). The resistance of the mutant stems from replacement of one of three invariant arginines (Arg 292→Lys) that are conserved among all viral and bacterial NAs and participate in the conformational change of sialic acid moiety necessary for substrate catalysis. The Lys292 mutant was selected in vitro after 15 passages at increasing concentrations of GG167 (from 0.1 to 1,000 μM), conditions that earlier gave rise to GG167-resistant mutants with a substitution at the framework residue Glu119. Both types of mutants showed similar degrees of resistance in plaque reduction assays, but the Lys292 mutant was more sensitive to the inhibitor in NA inhibition tests than were mutants bearing a substitution at framework residue 119 (Asp, Ala, or Gly). Cross-resistance to other NA inhibitors (4-amino-Neu5Ac2en and Neu5Ac2en) varied among mutants resistant to GG167, being lowest for Lys292 and highest for Asp119. All GG167-resistant mutants demonstrated markedly reduced NA activity, only 3 to 50% of the parental level, depending on the particular amino acid substitution. The catalytic mutant (Lys292) showed a significant change in pH optimum of NA activity, from 5.9 to 5.3. All of the mutant NAs were less stable than the parental enzyme at low pH. Despite their impaired NA activity, the GG167-resistant mutants grew as well as parental virus in Madin-Darby canine kidney cells or in embryonated chicken eggs. However, the infectivity in mice was 500-fold lower for Lys292 than for the parental virus. These findings demonstrate that amino acid substitution in the NA active site at the catalytic or framework residues, followed by multiple passages in vitro, in the presence of increasing concentrations of the NA inhibitor GG167, generates GG167-resistant viruses with reduced NA activity and decreased infectivity in animals.

Development of a potent anti-influenza drug that does not cause side effects and is effective against all virus strains is highly desirable. The neuraminidase (NA) of influenza viruses has been considered a suitable target for improved anti-influenza drugs because the activity of this surface protein is essential for virus spread (17, 19, 20). The NA is a receptor-destroying enzyme that catalyzes the hydrolytic cleavage of the α-ketosidic bond linking a terminal sialic acid to the adjacent carbohydrate moiety (21). The precise mechanism of this reaction is not completely understood, although the crystalline structure of the NA has been solved for several influenza viruses, as well as for complexes of the enzyme with its natural substrate (sialic acid) and with sialidase inhibitors (3, 6, 7, 26–29). The NA active site is a shallow pocket lined by a central shell of 11 strictly conserved amino acid residues, some of which bind directly to substrate and participate in catalysis, while others provide a structural framework (8).

Information gained from enzyme crystalline structures was used to design potent and selective inhibitors of the influenza virus NA. One of these inhibitors, 4-guanidino-Neu5Ac2en (GG167), shows activity against influenza viruses in vitro (12, 30, 34) and in vivo (12, 22, 30). The drug appears to be effective for both the prevention and early treatment of human influenza virus infection (15). The specific and potent inhibition of influenza NA activity by the compound is a result of the replacement of the hydroxyl group at the C-4 atom by the guanidino group in the structure of Neu5Ac2en, an inhibitor of sialidases. The interaction of guanidino group with two framework residues, Glu119 and Glu227, results in tight affinity of GG167 for the active site (30). Because the design of the inhibitor was based on the conserved nature of amino acids forming the enzyme active site, it becomes important to study resistant mutants of influenza viruses that arise due to inhibitor pressure and to determine which mutations in the NA gene are responsible for the mutants' resistant phenotype. Such information can be valuable in further efforts to improve drug design.

There have been no reports of the isolation of GG167 mutants in vivo; however, three resistant mutants have been generated in vitro: A/NWS-G70c (H1N9) (5, 24), A/turkey/Minnesota/80 (H4N2) (A/Ty/Mn) (13), and B/HK/8/73 (HG) (24). All of the GG167-resistant mutants described thus far have had the same amino acid residue replaced, the Glu at position 119. In the present study, we describe the isolation and characterization of a resistant mutant of A/Ty/MN whose NA contains a substitution at the catalytic residue (Arg to Lys at position 292) in the enzyme active site. Because the Arg at 292 plays an important role in substrate binding and is conserved among viral and bacterial NAs (see the review by Colman [9]),

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we reasoned that the enzyme properties of the selected mutant might differ substantially from those associated with replacement of a framework residue and therefore could be related to greater reductions of infectivity. Additionally, we provide the first information of the replication of catalytic and framework mutants in an animal model system and their physical stability in vitro.

MATERIALS AND METHODS

NA inhibitors. The two NA inhibitors used in this study, 4-guanidino-Neu5Ac2en (4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid) and 4-amino-Neu5Ac2en (30), were provided by the Glaxo Wellcome R&D, Stevenage, United Kingdom. Neu5Ac2en is commercially available.

Viruses and cells. The egg-grown influenza viruses (A/Ty/MN and A/NWS/1/33 (H1N1) were from the repository at St. Jude Children's Research Hospital. Viruses were propagated in 11-day-old embryonated chicken eggs and in Madin-Darby canine kidney (MDCK) cells. The MDCK cells were routinely passaged in Eagle's minimal essential medium (MEM) containing 5% fetal calf serum. Viruses used for NA assays were grown in embryonated chicken eggs, and allantoic fluid was clarified with low-speed centrifugation and purified by differential centrifugation through 20 to 70% sucrose gradient in a Beckman SW28 rotor at 20,000 rpm. After pelleting at 24,000 rpm, viruses were resuspended in Ca-containing phosphate-buffered saline (PBS; 6.8 mM $CaCl_2$ and 154 mM NaCl in 20 mM borate buffer [pH 7.2]) and stored at −70°C. Reassortants bearing the NA gene of avian origin (A/Ty/MN) and the HA from A/NWS/1/33 (H1N1) were prepared as described by Webster (33).

Generation of GG167-resistant mutants. The GG167-resistant mutant with an amino acid substitution at the catalytic residue 292 (Arg→Lys) was generated by a protocol similar to that described for the generation of mutants with a substitution at framework residue 119 (13), with slight modifications. MDCK cells were infected with egg-grown A/Ty/MN and cultured in the presence of GG167. Supernatant was harvested 3 days after infection. Virus yield that was present in the supernatants, as determined by hemagglutination of chicken erythrocytes, was used for the next passage in the presence of the inhibitor. Consequent passages were performed at the following drug concentrations: three passages at 1 μM, three at 10 μM, and two at 500 μM. Virus was cloned in MDCK cells by plaque formation in the presence of the drug (100 μM). Individual virus clones were passaged three more times in the presence of GG167, twice at 100 μM and once at 1,000 μM. Viruses were propagated in MDCK cells without GG167 and used for further analyses. Egg-grown A/Ty/MN was propagated in the same fashion in MDCK cells and used as control (parent).

Virus plaque assay. Plaque assays were performed as described by Hayden et al. (14), with minor modifications. Briefly, confluent MDCK cells were inoculated with influenza virus diluted in MEM containing 5% bovine serum albumin (BSA) and 1 μg per ml of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. Cells were incubated for 1 h at room temperature to permit virus adsorption and then overlaid with MEM containing 0.8% agarose, 4% BSA, 2 μg of TPCK-treated trypsin per ml, and 0.001% DEAE-dextran. After 3 days at 37°C, the agar overlay was discarded, and plaques were visualized by staining cells with 0.1% crystal violet containing 10% formaldehyde. A plaque reduction assay was performed by the same procedure, and GG167 was added to the agar overlay at different concentrations. The data from three experiments were pooled to determine the IC_{90} (drug concentration at which plaque numbers reduced to 10% of the controls value) and IC_{50} (drug concentration at which plaque size is reduced to 50% of the controls value).

NA enzyme assays. NA activity was measured as described by Warner and O'Brien (32), with 2 mM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-NANA) used as a substrate. Reaction mixtures containing purified virus (5 μl) and substrate (total volume of 10 μl in 0.1 M sodium phosphate buffer [pH 5.9]) were incubated in U-bottom microtiter plates at 37°C for 30 min (if not otherwise mentioned), at which time the reactions were stopped by addition of 200 μl of 0.1 M glycine buffer (pH 10.7) containing 25% ethanol. The fluorescence of released MU-NANA was determined with a Labsystems Fluoroskan spectrophotometer (excitation wavelength, 355 nm; emission wavelength, 460 nm). The NA activity was calculated and expressed as number of fluorescence units per microgram of total protein. Protein concentration in purified viruses was determined with a Bio-Rad protein assay. All reactions were carried out in duplicate; the mean values of three independent determinations were used for presentation of data.

To determine an pH optimum for each mutant's enzyme activity, the measurements were performed by a standard protocol in 0.1 M sodium acetate buffer (in a pH range of 4.0 to 5.9) and in 0.1 M sodium phosphate buffer (in a pH range of 5.9 to 8.0). The NA activity was expressed as a percent of maximum activity determined for both buffers.

The sensitivity of each mutant's NA to incubation at acidic pH was determined by adjustment of virus solutions (in Ca-containing PBS) to a pH range of 4.0 to 5.9 by using 0.1 M sodium acetate buffer on ice and then transferring the solutions to 37°C for 15 min. Solutions were placed on ice and brought to pH 5.9 by using 0.4 M sodium phosphate buffer. After treatment, virus solutions were subjected to standard NA assays in 0.1 M sodium phosphate buffer (pH 5.9).

To measure the inhibition of neuraminidase activity (NI), we mixed equal volumes of virus and 4-guanidino-Neu5Ac2en, 4-amino-Neu5Ac2en, or Neu5Ac2en and preincubated the solutions at room temperature for 30 min.

NA activity was also measured by the colorimetric assay (2), with fetuin as a substrate. Two trisaccharides, *N*-acetylneuraminic acid-α-2,3-galactose-β1,4 glucose [α-(2,3)-NANL] and *N*-acetylneuraminic acid-α-2,6-galactose-β1,4 glucose [α-(2,6)-NANL], were used as substrates in studies to determine NA specificity.

Kinetics of virus growth in vitro. MDCK cells were infected with viruses at a multiplicity of 0.001 PFU/cell. After 1 h of adsorption at room temperature, the cells were washed twice with PBS and placed in medium containing 4% BSA and 1 μg of trypsin-TPCK per ml. To determine the virus yield, we harvested fluids from cultures at different intervals postinfection, centrifuged them at 800 × *g*, and then assessed plaque formation.

Virus infection in mice. Female BALB/CBYJ mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Groups of three mice per virus dilution were anesthetized with methoxyflurane and exposed intranasally to 100 μl of PBS containing virus at 0.5-log dilutions. The infected mice were sacrificed at 3 days postinfection, and the lungs were harvested and homogenized. To detect virus replication in mouse lungs, we infected embryonated chicken eggs with lung homogenates and 2 days later tested the allantoic fluid by hemagglutination of chicken erythrocytes. The mouse 50% infectious dose (MID_{50}) is defined as number of PFU of virus required to infect 50% of the animals and expressed as antilog$_{10}$ of average results obtained in two experiments.

Sequencing of the NA gene. Viral RNA was prepared from cell-free supernatants, and reverse transcription-PCR products were sequenced by the fmol sequencing system (Promega) as described previously (13).

TABLE 1. Reduction of plaque formation of viruses in the presence of GG167

Virus	IC_{90} (μM) plaque no.	IC_{50} (μM) plaque size
Parent	0.1	0.01
Lys292	>1,000	10
Ala119	>1,000	100
R-parent[a]	1	0.5
R-Lys292	1,000	1
R-Ala119	1,000	10

[a] Reassortants (H1N2) were prepared between the parental virus (or mutant) and A/NWS/1/33 as described by Webster (33).

RESULTS

Isolation and characterization of GG167-resistant viruses. The NA inhibitor GG167 was designed to interact entirely within the NA active site (30). To determine which conserved amino acid residues could be substituted to provide resistance to the inhibitor, we performed two independent experiments under similar conditions. An avian influenza virus A/Ty/MN, was grown in vitro in the presence of increasing concentrations of GG167; virus variants whose growth was not inhibited at any drug concentration tested were considered GG167-resistant mutants. Fifteen passages of the viruses generated a variant with a substitution at catalytic residue 292 of the NA (AGA→AAA). Under similar conditions, 13 passages led to selection of virus variants with three different substitutions at framework residue 119 (13). The availability of mutants with substitutions in both the catalytic site (residue 292) and the framework residue (residue 119) permitted a comparison to evaluate the impact of these substitutions on resistance to the NA inhibitors, NA enzyme properties, and infectivity of the mutants in vitro and in vivo.

Resistance to NA inhibitors. (i) Plaque reduction assays. To evaluate the level of resistance to GG167 demonstrated by the mutant with a substitution at catalytic residue 292 (Lys292), we performed plaque reduction assays over the range of inhibitor concentrations in agar overlay. The demonstrated level of resistance (>1,000 μM) was similar to that for the mutant with a substitution at the framework residue (Ala119) (Table 1). However, the IC_{50} for Lys292 was 10-fold lower than that for

TABLE 2. Characterization of GG167-resistant mutants in NA inhibition assays

Virus	Resistance to NA inhibitor in NI assay (fold)[a]		
	4-Guanidino-Neu5Ac2en	4-Amino-Neu5Ac2en	Neu5Ac2en
Parent	1	1	1
Lys292	10	4	2
Asp119	2,500	200	20
Ala119	600	35	7
Gly119	700	3	10

[a] The NI assays were performed with purified viruses and MU-NANA as the substrate. The IC_{50}s of the parental virus were 6 nM, 11 μM, and 130 μM for 4-guanidino-Neu5Ac2en (GG167), 4-amino-Neu5Ac2en, and Neu5Ac2en, respectively.

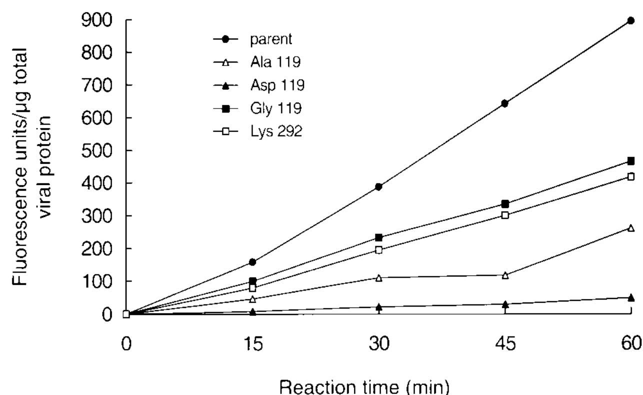

FIG. 1. Reduced virion NA activity of GG167-resistant mutants of A/Ty/MN. Purified virus was incubated at 37°C with MU-NANA as the substrate in 0.1 M sodium phosphate buffer (pH 5.9) for the indicated time periods. NA activity per microgram of total virus protein was estimated based on results of three experiments. Protein concentration was determined with a Bio-Rad protein assay kit. The fluorescence associated with release of MU-NANA was determined after reactions were stopped by addition of 0.1 M glycine buffer (pH 10.7) containing 25% ethanol.

Ala119, based on the observed reduction in plaque size. Hence, Lys292 was slightly more sensitive to the inhibitor than was Ala119 with respect to plaque production.

Because the hemagglutinin (HA) may contribute to resistance to the NA inhibitor in tissue culture, we made reassortant viruses that possessed an NA gene of A/Ty/MN origin and the HA from A/NWS/1/33 (H1N1). The reassortant possessing the NA with a substitution at the catalytic residue 292 (R-Lys292) demonstrated resistance in the plaque reduction assay, as did the R-Ala119 reassortant, indicating that the presence of the mutant NA is sufficient for resistance to GG167, even in a combination in which the HA derived from an untreated virus (NWS). Thus, similar levels of resistance to GG167 were generated by replacement of Arg at catalytic residue 292 and substitution at framework residue 119.

(ii) NI assays. We also compared the levels of GG167 resistance associated with a substitution at catalytic residue 292 versus one at residue 119. NA activity was measured after 30 min of treatment over a range of drug concentrations, and IC_{50}s were determined for each mutant. Substitution of Arg for Lys at catalytic residue 292 resulted in a 10-fold reduction of NA sensitivity to GG167, by comparison with the parental NA, when MU-NANA was used as the substrate (Table 2). By contrast, mutants defined by a substitution at framework residue 119 showed as much as a 2,500-fold reduction in sensitivity to the inhibitor. Similar results were obtained when a large substrate (fetuin) was used in the assays (data not shown).

Table 2 shows the results of studies to determine levels of resistance to another inhibitor, 4-amino-Neu5Ac2en, as well as Neu5Ac2en, the structural prototype for both compounds. All mutants were less sensitive to 4-amino-Neu5Ac2en and Neu5Ac2en than was the parental strain; however, none showed the large decreases in sensitivity seen in assays with GG167. Interestingly, the highest level of resistance was demonstrated by the mutant with an Asp at position 119, the amino acid residue considered to be conservative substitution for Glu. The Ala119 mutant showed essentially the same resistance as did the Gly119 mutant while displaying 10-fold less sensitivity to 4-amino-Neu5Ac2en. As expected, the sensitivity of the A/Ty/MN parental virus was highest to GG167 (IC_{50} = 6 nM), followed by 4-amino-Neu5Ac2en (IC_{50} = 11 μM) and then Neu5Ac2en (IC_{50} = 130 μM).

Taken together, the results of NI tests demonstrated that replacement of Arg by Lys at catalytic residue 292 does not cause a large reduction in sensitivity of each mutant's NA to GG167 or to the prototype inhibitor Neu5Ac2en. Mutants defined by substitutions at framework residue 119 appear more resistant than Lys292 to the three NA inhibitors tested. Mutants were consistently more resistant to GG167 and less resistant to another Neu5Ac2en derivative, 4-amino-Neu5Ac2en. The level of resistance shown by viruses with substitutions at framework residue 119 depended on the amino acid at that position, Asp being associated with the greatest reduction in sensitivity.

NA specific activity of the resistant mutants. The isolation of GG167-resistant mutants in vitro with substitutions in the NA active site raised questions about the NA activities of these viruses. At standard conditions for measurement of influenza virus NA activity, all resistant mutants showed less activity than the parental virus (Fig. 1), with the level of reduction varying widely, from 50% for Lys292 to 3% for Asp119. To rule out the possibility that substitutions in the NA active site resulted in a change of enzyme specificity for the linkage between terminal sialic acid and penultimate sugar, we performed assays with two trisaccharides, α-(2,3)-NANL and α-(2,6)-NANL. Both the parental virus and the GG167-resistant mutants had a preference for the α-(2,3) linkage (results not shown).

To determine if the substitutions in the enzyme active center affected the pH optimum of NA activity, their reactivities were studied over the pH range from 4.0 to 8.0. The NA activities of the parental and mutants with a substitution at framework residue 119 were similar at different pHs, with an optimum of pH 5.9; in contrast, the pH optimum for the catalytic mutant (Lys292) was shifted to 5.3 (Fig. 2). The other feature of this mutant was a decline in the level of NA activity in the physiological pH range. However, no difference was detected in the level of enzyme sensitivity to GG167 when the NI assays were done at pH 5.3 (optimum) rather than 7.4.

We also wanted to know if the parental and mutant NAs differ in enzyme stability during incubation at low pH. Virus preparations were treated for 15 min at 37°C over the pH range from 4.0 to 5.9, and NA activity was measured after adjustment to pH 5.9 (Fig. 3). Mutants with substitutions at residue 119 behaved similarly and demonstrated a significant reduction in NA activity at low pH compared with the parent. The NA activity of the catalytic mutant was less sensitive to low pH treatment than those of the framework mutants but more sensitive than the parental enzyme activity.

The NA of the GG167-resistant mutant with a substitution at position 119 (Glu→Gly) was recently shown to be less stable than the parental virus even at 4°C (18). This finding prompted

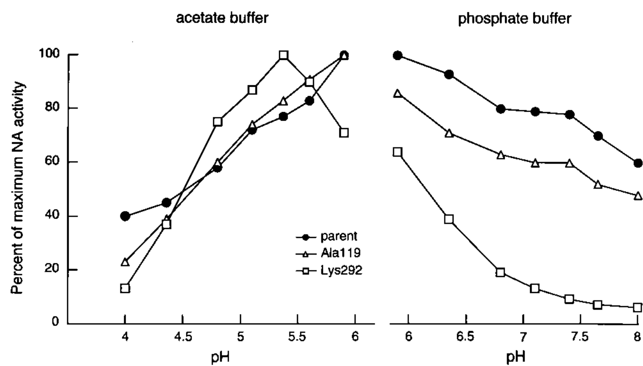

FIG. 2. Effects of mutations in the enzyme active site on pH optimum. Reactions were done in 0.1 M sodium acetate buffer (pH 4.0 to 5.9) and in 0.1 M sodium phosphate buffer (pH 5.9 to 8.0). Incubation time was 30 min. NA activity is expressed as a percentage of maximum activity for each virus in either buffer system.

us to measure the thermostability of each type of mutant selected in our study. The viruses were incubated at 4, 37, and 42°C for 48 h in PBS containing 1% BSA. The enzyme activities of the parental virus A/Ty/MN and its GG167-resistant mutants were not reduced after incubation at 4°C and were also remarkably stable at 37 and 42°C for the parent and the catalytic mutant. The NA activity of mutants with the substitution at framework residue 119 was less stable at elevated temperatures and was up to 40% less than the initial activity.

Replications of the mutants in tissue culture and in embryonated chicken eggs. We also wished to know if the selection of a GG167-resistant phenotype had affected the ability of the viruses to propagate in vitro. Thus, MDCK cells were infected at a low multiplicity of infection (0.001), and virus yields were titrated at different intervals (Fig. 4). The mutant with substitution at catalytic residue 292 grew to slightly higher titers than the parent; however, there were only modest differences among the growth properties of the mutants overall (data shown for two mutants). In the consequent comparison, GG167-resistant mutants with substitutions at catalytic residue 292 or framework residue 119 produced virus yields in another cell culture system, embryonated chicken eggs, similar to the yield of the parental virus (infectivity titer, 0.2×10^7 to 0.8×10^7). Thus, viruses with equal or slightly higher growth potential were selected under pressure of the GG167 inhibitor, and mutations in the active site of the NA did not affect their ability to replicate in vitro.

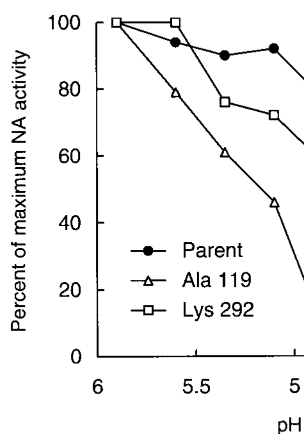

FIG. 3. Stability of mutant NA to low-pH treatment. Viruses were treated over a pH range from 4.0 to 5.9, using 0.1 M sodium acetate buffer (abscissa). After incubation for 15 min at 37°C, the reaction mixtures were brought to pH 5.9 with 0.4 M sodium phosphate buffer, and NA activity was measured by the standard procedure. Results are expressed as a percentage of maximum activity for each virus.

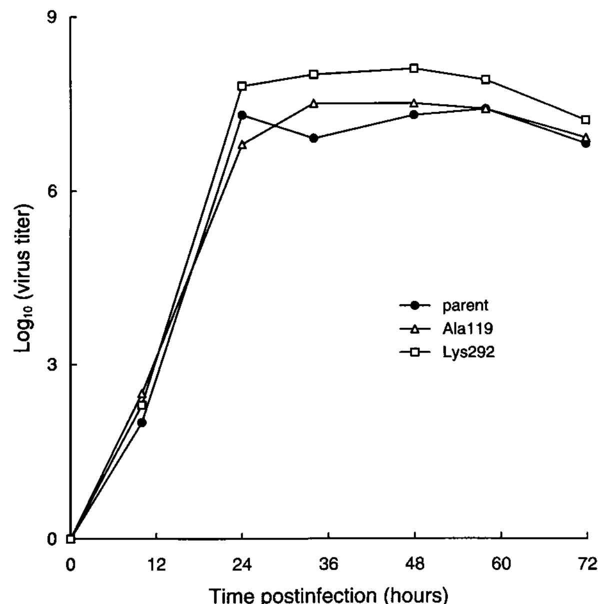

FIG. 4. Replication of the parental A/Ty/MN virus and GG167-resistant mutants in MDCK cells. Cells were infected with the parent, Lys292, and Ala119 at a multiplicity of 0.001 PFU per cell and incubated at 37°C. At the indicated times postinfection, supernatants fluids were harvested, and the virus titer was determined in plaque assays. Each experiment was repeated twice, with similar results. The data from one representative experiment are shown.

Replication of GG167-resistant mutants in vivo. The in vitro replication of our GG167-resistant mutants was not affected; however, we wanted to know if their infectivity was reduced in animals. To test this hypothesis, we infected mice with the parental and mutant viruses and assayed viral replication in lungs 3 days postinfection. In contrast to the results obtained in tissue culture, the Lys292 mutant demonstrated attenuation in mice (MID_{50} = 3.4) compared to the parent (MID_{50} = 0.6); that is, about 500 times more PFU of the Lys292 mutant was required to cause infection in 50% of inoculated animals. The Ala119 mutant, with the substitutions at the framework residue, also showed slightly reduced infectivity in mice (MID_{50} = 1.4).

DISCUSSION

Here we demonstrate, apparently for the first time, the emergence of an influenza virus with a substitution at the catalytic residue of the NA active site in the presence of a neuraminidase inhibitor. The mutant, in which Lys replaced Arg at position 292, was isolated after 15 passages in the presence of increasing concentrations of GG167 (from 1 to 1,000 μM). Under similar conditions, we isolated three other mutants with different amino acid substitutions at position 119 (13). Isolation of GG167-resistant mutants with Gly at position 119 was also reported recently by Blick et al. (5) and Staschke et al. (24) when a slightly different protocol for selection was used. The intent of these isolations and subsequent characterizations was to provide additional information about the func-

tion of influenza NA that could be used to improve the design of antiviral compounds that target the viral neuraminidase.

The design of GG167 was based on utilization of the previously unoccupied pocket of the influenza virus NA, where the guanidino group of the inhibitor interacts with the framework residues Glu119 and Glu227 (30). Although framework residues are conserved among all influenza virus strains, their functions are restricted to providing structural support for the catalytic residues that participate in substrate hydrolysis. Hence, the isolation of GG167-resistant mutants with amino acids substitutions at framework residue 119 was not totally unexpected although it required multiple passages in the presence of GG167.

Less predictable was the isolation of GG167-resistant mutants with a substitution at the catalytic residue of the NA active site, under conditions similar to those used to generate the framework mutants. Three arginines, Arg118, Arg292, and Arg371, form an arginine pocket in the enzyme active site. The function of this triad is to bind to the substrate through the carboxylate group and facilitate the conformational change of the pyranose ring of the substrate from the axial position into a pseudo-equatorial position (25). This conformational change is believed to be very important for catalysis of the substrate. Although the most important arginine occupies position 371, all three of these residues have been conserved in the viral and bacterial neuraminidases studied to date (8, 10).

The mutant with a substitution at catalytic residue 292 demonstrated the same level of resistance in plaque reduction assays in the presence of GG167 as did mutants with a substitution at framework residue 119 (>1,000 μM). Despite the high level of resistance demonstrated for Lys292 in plaque assays in the presence of GG167, the NA of this mutant was more sensitive to GG167 in NI assays than were mutants carrying substitutions at framework residue 119. Lys292 was also the most sensitive to two other inhibitors, 4-amino-Neu5Ac2en and Neu5Ac2en. The lack of correlation between the inhibitory effect of GG167 on enzyme activity and virus plaque formation was also described for human influenza virus isolates (34). These viruses had different levels of sensitivity to GG167 in plaque assays despite similar sensitivities of their neuraminidases to the inhibitor. Both our observations and those given above (34) may be a reflection of a difference in the level of NA activity necessary for virus spread from cell to cell in tissue culture. The need for a functioning NA may vary among virus strains depending on the affinity of the HA for cell receptors. These and other factors may contribute in the lack of direct correlation between sensitivity of the enzyme to the drug and the level of inhibition of virus growth in the presence of the NA inhibitor in the medium.

Comparison of the framework mutants Asp119, Ala119, and Gly119 in NI assays revealed a different profile of resistance to the NA inhibitors, depending on the specific amino acid substitution for the conserved Glu at 119. Thus, Gly119 was more sensitive to 4-amino-Neu5Ac2en than was Ala119, but their reductions in sensitivity to the prototype inhibitor Neu5Ac2en were comparable. This result correlates well with data obtained by Blick et al. (5), demonstrating significant reduction in the sensitivity of Gly119 to GG167 but full sensitivity to 4-amino-Neu5Ac2en.

Unexpectedly, the most resistant of all three mutants was Asp119. Substitution of Asp for Glu is generally considered a conservative change, in that Asp carries the negative charge as Glu does but it has a shorter side chain. In two other mutants, Glu was replaced with Ala or Gly, which do not have a negative charge and have side chains shorter than Asp's. Analyses of the crystalline structure of the mutants' NA could provide a detailed geometry of their active sites and explain the difference in their levels of resistance to the inhibitors.

Since the GG167 resistance was a result of substitutions at the strictly conserved amino acid residues of the NA active site, it was important to investigate if these substitutions affected enzyme specific activity. Indeed, replacement of Arg with Lys at position 371 (arginine triad) of the expressed NA protein led to a significant (more than 90%) reduction of enzyme activity (16). In the present study, the replacement of another Arg of this triad (at position 292) with Lys did not cause a significant reduction of enzyme activity measured by a standard procedure at pH 5.9 but led to a switch in pH optimum from 5.9 to 5.3. Similar changes in the properties of a recombinant NA where the conserved His274 was replaced with Tyr or Asn have been described (1, 16). With respect to framework mutations, recently Blick et al. (5) estimated the NA activity of a GG167-resistant mutant (Glu→Gly substitution at position 119) to be equal to the parental activity, while Staschke et al. (24) reported the same activity as being only 5% of the parental level. Other evidence for reduction of the enzyme activity due to substitutions at position 119 was reported for the influenza virus NA protein expressed on the surface of mammalian cells (11). Thus, the issue of whether development of GG167 resistance is accompanied by reduction in NA activity was unclear. Measurement of the NA activity of the mutants required standardization of the virus preparations for total protein content and for the content of NA molecules on the virus particles. Despite all precautions, errors may still be introduced in the estimation of NA activity. Another issue that must be taken in consideration is the relative instability of mutant NA under different conditions of incubation. Thus, dramatic difference in the stability of the mutant NA (Gly119) under prolonged incubation at high and low temperatures was recently reported (18). Our data support the notion that resistance to GG167 is accompanied by impairment of NA function of mutant viruses due to a reduction of the NA activity as well as relative instability of the mutants' NA at low pH and elevated temperature.

Nonetheless, even with reduced NA activity, GG167-resistant mutants were able to successfully replicate in vitro. Both the mutant with the substitution at catalytic residue 292 and the one with a substitution at framework residue 119 grew to titers the same as or slightly higher than that of the parental virus in MDCK cells and embryonated chicken eggs. These results are in accord with a recent report (24) that mutants with Gly at position 119 grew as well as or better than wild-type viruses. Thus, reduced NA activity does not preclude virus growth in vitro, suggesting that the HA gene or perhaps other genes may compensate for the impaired NA function at same stage of virus release from the cell surface. In this respect, multiple passages in culture which are required for selection of GG167-resistant mutants may enrich the virus population with compensatory mutations in other genes.

Although the growth of GG167-resistant mutants was not impeded in tissue culture, higher doses of mutants than of the parental virus were required to infect animals. Attenuation in mice of the virus bearing a mutation at catalytic residue 292 is likely to be a result of a significant reduction in the NA activity at physiological pH demonstrated for this mutant. Impairment of NA function may cause an entrapment of inoculated virus in the upper respiratory tracts of animals, leading to attenuation of the virus in vivo. However, we cannot rule out that attenuation of Lys292 is not solely due to this mutation but may be a complex phenotype involving changes in the HA molecule or other gene products, which make replication of this mutant in mice less efficient. Thus, when the virulence of amantadine-resistant influenza virus mutants was studied in chickens, all of

them were partially to fully virulent, and the level of virulence varied among strains even with identical mutations in the M2 protein (4).

The isolation of a GG167-resistant mutant with a substitution at catalytic residue 292 raises the question of cross-resistance to other NA inhibitors whose designs are based on enzyme structure. Recently, White et al. (31) described a sialic acid-derived phosphonate analog that acts by strengthening of the charge-charge interaction with the arginine pocket in the NA active site. Whether substitution at one of the three arginines within the active site influences the sensitivity of the NA mutant to this analog is a worthwhile question for future research.

In conclusion, the necessity for multiple passages of influenza viruses in vitro in the presence of inhibitor in order to obtain resistant viruses, reduction in specific activity, and relative instability observed for the mutants' NA indicates the correct and successful approach for the NA inhibitor's design and suggests that resistant mutants will not be readily isolated in human trials but must be diligently pursued.

ACKNOWLEDGMENTS

We thank Clayton Naeve and the St. Jude Children's Research Hospital (SJCRH) Center for Biotechnology of preparation of oligonucleotides and computer support. We are also grateful to Aarnoud C. Van Der Spoel and the Department of Genetics (SJCRH) for assistance with the NA assay and for use of their Fluoroskan spectrophotometer. We thank M. N. Matrosovich, a visiting scientist in SJCRH, for valuable discussion, Chris Clark and Daniel Wehr for excellent technical assistance, and John Gilbert for editorial work.

This work was supported by Public Health Service research grant AI-08831 from the National Institute of Allergy and Infectious Diseases, Cancer Center Support (CORE) grant CA-21765, and the American Lebanese Syrian Associated Charities.

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