 Selection of Influenza A and B Viruses for Resistance to 4-Guanidino-Neu5Ac2en in Cell Culture

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The reassortant influenza viruses, A/NWS-G70c with N9 neuraminidase (NA) and B/HK/8/73 (HG) with B/Lee/40 NA, were selected for resistance to 4-guanidino-Neu5Ac2en (4-GuDANA) by passing the virus in stepwise increases in the concentration of 4-GuDANA. In the NA of resistant viruses, the absolutely conserved Glu 119, which lies in a pocket beneath the active site of the enzyme and interacts with the guanidinium moiety of 4-GuDANA, was changed to Gly. The mutant NA was >200-fold more resistant to 4-GuDANA than was the wild-type enzyme. During 72 h in cell culture, resistant A and B viruses displayed much less NA activity than did wild-type viruses but did undergo multicycle replication. While emergence of resistance to 4-GuDANA has not been observed in vivo, these results demonstrate that the development of resistance is possible and can be mediated by a single amino acid change in the active site of the viral NA.

Neuraminidase (NA) is one of the two envelope glycoproteins encoded by both influenza A and B viruses. This enzyme catalyzes the removal of terminal sialic acid residues from viral and cellular glycoconjugates, thus facilitating the release of the virus from the infected cell and its spread through the mucus of the respiratory tract. The first X-ray crystallographic structure of influenza A virus NA was determined >10 years ago [1], and other NA structures have been elucidated since then. The catalytic sites of the NA from all influenza A and B viruses so far studied are structurally very similar [2, 3], and several amino acids that line the active site of the enzyme and participate in the binding and catalysis of the substrate are invariant, as reviewed in [4]. Structure-based design of active site inhibitors of influenza virus NA led to the identification of 4-guanidino-2-deoxy-2,3-didehydro-D-N-acetylneuraminic acid (4-GuDANA) as a potent and selective inhibitor of this enzyme [5] and viral replication in vitro and in vivo [5–7], as well as in humans [8]. It was of interest to determine whether viruses resistant to 4-GuDANA could be selected in vitro.

Results

In liquid culture, MDCK cells infected with either resistant influenza A or B virus showed extensive cytopathic effect in MDCK cells in the presence of 5 μg/mL 4-GuDANA, whereas MDCK cells infected with parent virus in the presence of the same concentration of 4-GuDANA showed no cytopathic effect [11]. Using a standard plaque assay for influenza [12], 4-GuDANA inhibited parent influenza A/NWS-G70c virus by 50% at a concentration (IC50) of 0.2 μg/mL but inhibited resistant influenza A virus at an IC50 of 4.6 μg/mL. Similarly, 4-GuDANA inhibited parent influenza B/HK/8/73 (HG) at an IC50 of 0.07 μg/mL but inhibited the resistant virus at an IC50 of 6.0 μg/mL [11].

The complete nucleotide sequences of NA and hemagglutinin (HA) genes from parent and from 4-GuDANA–resistant A/NWS-G70c and B/HK/8/73 (HG) viruses were determined using synthetic oligonucleotide primers according to standard procedures [13, 14]. The NA gene of the 4-GuDANA–resistant A/NWS-G70c (N9) virus was found to contain a single mutation (nucleotide 377) leading to the replacement of glutamate with glycine 119 [11]. Similarly, the NA gene of the 4-GuDANA–resistant B/HK/8/73 (HG) also contained a single mutation (nucleotide 403) leading to the amino acid substitution of glutamate with glycine at position 117 [11]. This glutamic acid lies in a pocket beneath the active site of the enzyme [1, 5, 15] and is absolutely conserved among all known influenza virus NAs. These results are consistent with recent structural findings that
demonstrate a strong electrostatic interaction between the carboxylate of glutamate 119 and the C-4 guanidinium group of 4-GuDANA [5, 15]. The replacement of glutamate 119 with glycine points to the importance of this interaction as the basis for the inhibitory activity of this sialic acid analog.

There were no differences between the HA gene of the parent A/NWS-G70c virus and that of the 4-GuDANA–resistant mutant. In contrast, two mutations were observed in the HA gene from 4-GuDANA–resistant B/Hong Kong/8/73 (HG): one at nucleotide position 489 and the other at nucleotide position 504, both resulting in a codon change of AAC to AGC, yielding changes of asparagine to serine at amino acids 145 and 150 [11]. This suggests that, at least for this type B virus, alterations in the HA in response to the selective pressure of 4-GuDANA may play a role in resistance to this inhibitor. Since asparagine 145 is part of an Asn-X-Ser/Thr consensus sequence for N-linked glycosylation, the change of this amino acid to serine would eliminate this glycosylation site. Because of the change from asparagine to serine at amino acid 150, asparagine 148 becomes a potential site for the attachment of a glycosyl group. If this is indeed the case, the new location of the carbohydrate moiety (at asparagine 148) in the HA of 4-GuDANA–resistant B/HK/8/73 (HG) virus could conceivably affect the availability or the affinity (or both) of this envelope glycoprotein for its sialic acid receptor. Alternatively, alterations in the glycosylation pattern of HA might affect the cleavability of HA by host-cell protease(s), a step that is essential for entry of influenza virus into the host cell [16]. Because of the role of NA in the cleavage of HA by cellular protease(s) [17, 18], the alteration in HA of the 4-GuDANA–resistant virus might be a compensatory response to the greatly reduced NA levels associated with this virus. The relevance of these changes in HA is not clear because they were not observed in the 4-GuDANA–resistant A/NWS-G70c virus [11] or in influenza B/Lee/40 virus selected for resistance to 4-GuDANA. Nevertheless, others [19, 20] have found that 4-GuDANA–resistant influenza A viruses contain an altered HA with changes that appear to affect receptor binding, thus supporting a role for HA in the development of resistance to this inhibitor.

When the activity of virion-associated NA from parent or 4-GuDANA–resistant virus was assayed, it was determined that enzyme activity from resistant A or B viruses was >200-fold more resistant to inhibition by 4-GuDANA than was enzyme activity from parent viruses [11]. In further experiments, NA heads, which contain only the tetrameric active site regions of the enzyme and not the stalk or membrane-anchoring region, were prepared by pronase digestion of virus obtained from embryonated chicken eggs and purified by sucrose centrifugation [9]. When normalized for protein content, the enzyme from resistant influenza A or B viruses displayed much less NA activity for either fetuin or the fluorogenic substrate, methylumbelliferyl-acetylmuraminic acid. The resistant A enzyme had ~5% of the parent level of enzyme, while the resistant B enzyme had <1% of the parent level [11]. Both viruses contained NA protein in amounts equivalent to those found in parent viruses as determined by the following experiment.

MDCK cells were infected with either parent influenza A or B virus or 4-GuDANA–resistant A or B virus. When cytopathic effect was extensive (at ~72 h), virus in the cell culture fluid was purified by sucrose-cushion centrifugation, denatured, and digested overnight with peptide:N-glycosidase F (according to the protocol of Garcia-Sastre A and Palese P, personal communication). This allows visualization of the NA protein by polyacrylamide gel electrophoretic analysis and Coomassie blue staining. Influenza A and B viruses, which were resistant to 4-GuDANA, contained amounts of NA protein equivalent to those seen in parent A and B viruses when normalized to total viral protein content. In contrast to our results, it has been recently observed that NA from parent and 4-GuDANA–resistant influenza A/NWS-G70c N9 did not differ in specific activity [21]. The investigations discussed here do not address the possibility that mutant NA is much more labile to the purification procedures used than is parent enzyme, thus making enzymatic activity of resistant enzyme much more difficult to detect. Now, recently available data confirm that the single amino acid change leading to 4-GuDANA resistance (E119G) leads to instability of the enzyme [22] and, in particular, a destabilization of the tetrameric structure (Colacino JM, et al., unpublished data).

It was demonstrated that 4-GuDANA–resistant A or B viruses were able to undergo multicyle replication in MDCK cells over 72 h [11] and, over this time period, expressed much lower levels of NA activity than did parent A or B viruses. These results indicate that the reduced levels of NA activity displayed by the 4-GuDANA–resistant viruses were sufficient to allow multicyle replication in tissue culture. Similarly, an influenza A/WSN/33 virus with 10% of the wild-type NA activity was capable of multiple rounds of replication in MDCK cells [17]. However, the absolute lack of NA results in the aggregation of virus particles at the infected cell surface and the inability to undergo multiple rounds of replication [23].

The influenza A and B viruses selected for resistance to 4-GuDANA appeared to be stable because they retained the drug-resistant phenotype even after 10 passages in cell culture in the absence of inhibitor. Additionally, 4-GuDANA–resistant influenza A/NWS/G70c retained the drug-resistant phenotype after passage through ferrets. Sequence analysis of the NA gene from this virus after isolation from a ferret demonstrated that the single nucleotide change leading to the E119G amino acid alteration was present. Hence these results indicate that should 4-GuDANA–resistant influenza virus emerge as a result of treatment, a population of resistant viruses that are transmissible might be generated. A precedent for this scenario was established for rimantadine treatment when it was observed that drug-resistant influenza emerged during the treatment of 5 family members and spread to other family contacts, resulting in typical influenza disease [24].
Discussion

Influenza A and B viruses were selected for resistance to 4-GuDANA in vitro and were shown to contain a single mutation in the NA gene segment, leading to the E119G or E117G changes, respectively. The glutamate at this position participates in a strong electrostatic interaction with the C-4 guanidinium group of 4-GuDANA. The resistant viruses had much less NA activity than did the parent viruses yet contained equivalent amounts of NA protein. The low activity of the isolated NA purified away from the virus appears to be a result of a destabilization of the enzyme due to the single amino acid change, E119G, in the active site. The NA of these viruses was resistant to 4-guanidino-Neu5Ac2en. Finally, both resistant viruses were able to undergo multicycle replication in MDCK cells. The drug-resistant phenotype and genotype appear to be stable, as determined in cell culture and in the ferret model of infection.

Although influenza virus resistant to 4-GuDANA could be selected in tissue culture experiments, whether such resistance can emerge in vivo remains to be determined. In preliminary experiments, the emergence of resistance to 4-GuDANA could not be demonstrated in mice infected with influenza and treated with this inhibitor [5]. In the clinical setting, an analysis of viruses collected from volunteers experimentally infected with influenza A/Texas/91 (H1N1) or from patients with naturally acquired influenza A (H3N2) before and after treatment with 4-GuDANA demonstrated that 4-GuDANA does not readily select for resistant influenza virus variants [8]. If the emergence of clinical resistance can be demonstrated using larger patient populations, whether such resistance will be due to alterations in the viral HA, NA, or both, and whether resistant viruses will be stable and transmissible will be questions of considerable interest for future approaches to antinfluenza chemotherapy.

References