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# Mutations in Human Parainfluenza Virus Type 3 Hemagglutinin-Neuraminidase Causing Increased Receptor Binding Activity and Resistance to the Transition State Sialic Acid Analog 4-GU-DANA (Zanamivir)

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**Entry and fusion of human parainfluenza virus type 3 (HPF3) require the interaction of the viral hemagglutinin-neuraminidase (HN) glycoprotein with its sialic acid receptor. 4-GU-DANA, a potent inhibitor of influenza virus neuraminidase, inhibits not only HPF3 neuraminidase but also the receptor binding activity of HPF3 HN and thus its ability to promote attachment and fusion. We previously generated a 4-GU-DANA-resistant HPF3 virus variant (ZM1) with a markedly fusogenic plaque morphology that harbored two HN gene mutations resulting in amino acid alterations. The present study using cells that express the individual mutations of ZM1 HN shows that one of these mutations is responsible for the increases in receptor binding and neuraminidase activities as well as the diminished sensitivity of both activities to the inhibitory effect of 4-GU-DANA. To examine the hypothesis that increased receptor binding avidity underlies 4-GU-DANA resistance, parallel studies were carried out on the high-affinity HN variant virus C22 and cells expressing the C22 variant HN. This variant also exhibited reduced sensitivity to 4-GU-DANA in terms of receptor binding and infectivity but without concomitant changes in the neuraminidase activity of HN. Another high-affinity HN variant, C0, was not resistant in terms of infectivity; however, a small increase in the receptor binding activity of C0 HN and a partial resistance of this activity to 4-GU-DANA were revealed by sensitive methods that we developed. In each virus variant, one mutation in HN accounted for both increased receptor binding avidity and 4-GU-DANA resistance; the higher affinity for the receptor overcomes the inhibitory effect of 4-GU-DANA. Thus, in contrast to influenza viruses for which 4-GU-DANA escape variants include hemagglutinin mutants with decreased receptor binding avidity that promotes virion release, for HPF3, HN mutants with increased receptor binding avidity are those that can escape the growth inhibitory effect of 4-GU-DANA.**

Human parainfluenza virus type 3 (HPF3) infection is initiated through two glycoproteins that project from the surface of the virion, hemagglutinin-neuraminidase (HN) and the fusion (F) protein. HN, which recognizes sialic acid-containing receptors, is responsible for binding the virus to the cell and for promoting F protein-mediated fusion. An additional function of HN, facilitating the spread of infection, comes from its neuraminidase (NA) activity, which cleaves the receptors whereby progeny virions aggregate on the cell surface and thus increases the number of free virions available for infecting additional cells (11, 14).

4-GU-DANA, a potent inhibitor of the influenza virus NA molecule (25) and an effective anti-influenza agent (7, 18), has been used in previous investigations designed to analyze the functions of HN. Greengard et al. and Levin Perlman et al. showed that 4-GU-DANA and related sialic acid analogs inhibit not only the NA activity but also the receptor binding activity of HN and thus its ability to promote attachment and fusion (6, 15). The demonstration that a single compound can block both HPF3 HN′s receptor binding and receptor cleaving activities was consistent with subsequently presented crystallographic studies on the HN of Newcastle disease virus (NDV), which suggested that a single active site provides both the receptor binding and hydrolytic functions (4). However, since there is no crystallographic information for HPF3 HN, the existence of two sites remains a possibility; in that case, the dual effect of 4-GU-DANA may be attributed to its having affinity for both sites, which would be in accord with the fact that recognition of the sialic group is necessary for both the hydrolytic and the binding activities.

To further address the mechanism of 4-GU-DANA action in HPF3 infection, we used 4-GU-DANA to select for escape variant viruses in vitro. A 4-GU-DANA-resistant HPF3 virus variant (ZM1) thus generated exhibited a markedly fusogenic plaque morphology and harbored two HN gene mutations resulting in the amino acid alterations T193I and I567V (20). Another HPF3 variant studied in parallel, C0 (19), also possessed an alteration at T193 (T193A) and exhibited similar plaque morphology but was not resistant to 4-GU-DANA according to the assays available at the time. ZM1 had greatly reduced sensitivity to 4-GU-DANA relative to the wild-type (WT) HPF3 and the C0 variant in terms of both its NA activity and its ability to bind sialic acid receptors, and it also retained infectivity in the presence of 4-GU-DANA.

The 4-GU-DANA-resistant variant ZM1 now provides us with a tool for dissecting HN functions and for elucidating the mechanism of 4-GU-DANA resistance. The present study us-

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ing cells that express the individual mutations of ZM1 HN shows that the mutation at residue 193 is responsible for all the deviations from the WT in the properties of ZM1. These properties include increases in receptor binding and NA activities as well as decreases in sensitivity of both activities to the inhibitory effect of 4-GU-DANA.

To examine the hypothesis that increased receptor binding avidity underlies ZM1's 4-GU-DANA resistance properties, parallel studies were carried out on the high-affinity HN variant C22 (19). Indeed, this variant also exhibited reduced sensitivity to 4-GU-DANA in terms of receptor binding and infectivity but without concomitant changes in the NA activity of HN. Another HN variant, C0, which like C22 arose during growth in the presence of exogenous NA, was not resistant in terms of infectivity. However, a small increase in the receptor binding activity of the HN variant C0 and a partial resistance of this activity to 4-GU-DANA were revealed by the sensitive methods developed in the present study. The results showed that in each virus variant, one mutation in HN (the site or substituted amino acid differed among the variants) was responsible for both the increased receptor binding avidity and the 4-GU-GANA resistance of the variants.

We have shown that 4-GU-DANA competes for the receptor, promoting rather than inhibiting the release of virus (22), and inhibits the binding of virus (6). Among the possible mechanisms for resistance to 4-GU-DANA, we favor the hypothesis that the mutant HNs have higher affinity for the receptor (and thus overcome the effect of 4-GU-DANA). In this paper we show that the characterization of several 4-GU-DANA-resistant variants supports this hypothesis.

## MATERIALS AND METHODS

**Cells and virus.** CV-1 (African green monkey kidney) cells were grown in Eagle's minimal essential medium (Mediatech Cellgro) supplemented with L-glutamine, antibiotics, and 10% fetal bovine serum (Sigma, St. Louis, Mo.) in 5% $CO_2$. 293T (human kidney epithelial) cells were grown in Dulbecco's modification of Eagle's medium (DMEM; Mediatech Cellgro) supplemented with 10% fetal bovine serum and antibiotics. WT virus stock was prepared by infecting CV-1 cell monolayers at a multiplicity of infection of 0.1 as described previously (15). Variant virus stocks were made in CV-1 cell monolayers from virus that was plaque purified three times. Virus titers were determined by plaque assay in CV-1 cells as described previously (15).

**Chemical.** 4-GU-DANA was prepared from Relenza Rotadisks (each containing 5 mg of zanamivir with lactose). A 50 mM stock solution was prepared by dissolving each 5-mg blister capsule in 285 μl of serum-free medium. Stock solutions were stored at −20°C.

**Plaque reduction assays.** The effect of 4-GU-DANA on plaque number was assessed by plaque reduction assays as described previously (15). Briefly, CV-1 cell monolayers ($1.5 \times 10^5$ cells in each well of a 24-well plate) were inoculated with 100 to 200 PFU of the WT or the C22, C0, or ZM1 variant. After a 90-min adsorption period, the inoculum was aspirated, the monolayers were washed, and an agarose overlay was added to the dishes. The plates were inverted and incubated at 37°C for 24 h. After the removal of the agarose overlay, the cells were immunostained for plaque detection (15) and plaques were counted under a dissecting stereoscope.

**Mutagenesis.** HN genes carrying sequence alterations in the regions coding for amino acid residues 193 and 567 were generated through the use of the PCR-based approach of overlap extension (8, 10). In order to introduce mutations in a site-directed manner, three PCRs were employed with primers designed against the WT HN. We used three external primers (ECORI; 5′ CCGGAATTC TCGAATACTGGAAGCACACCAATC [forward primer], SACI; 5′ GGGAG CTCATGGAATACTGGAAGCAACCAATC [forward primer], and BAMHI; 5′ CGCGGATCCGCGCTTAACTGCAGCTTTTTGGAATC [reverse primer]) carrying restriction enzyme sites for cloning into the pEGFP-C3 vector (BD Biosciences Clontech, Palo Alto, Calif.) along with two pairs of internal primers (T193I, 5′ GTTGATGGCTGTGTTAGAATTCCGTCCTTAGTTATAAATG [forward primer] and 5′CATTTATAACTAAGGACGGAATTCTAACACAGC CATCAAC [reverse primer], and I567V, 5′ CCTATGTTGTTCAAAACAGA GGTACCAAAAAGCTGCAG [forward primer] and 5′ CTGCAGCTTTTTGG TACCTCTGTTTTGAACAACATAGG [reverse primer]) designed to introduce the desired mutations along with unique restriction sites to permit rapid identification of introduced mutations. Two reactions employing one external primer and one internal mutagenesis primer were performed; the resulting products were gel purified and used as templates for the third PCR. The success of the third reaction relied on sequence overlap between the products of the first two reactions, and the third reaction resulted in a full-length product carrying the desired mutation. The success of the mutagenesis was determined by digesting the final PCR products with the appropriate restriction enzymes.

**HN and F constructs.** Mutagenized HN cDNAs were digested with either *Eco*RI and *Bam*HI or *Sac*I and *Bam*HI and ligated into digested pEGFP-C3 mammalian expression vector. pUC19 vector (BD Biosciences Clontech) containing a WT F cDNA insert was used as the template DNA for PCR amplification of the WT F cDNA with primers (*Xho*I/F/pCAGGS.MCS forward primer, 5′ CCCTCGAGGACCATGCCAACCTCAATACTGC, and *Bam*HI/F/pCA GGS.MCS reverse primer, 5′ CCCGGATCCTTTGTTTGTTAATACATAT GG) designed for ligation into the pCAGGS.MCS expression vector (21). Positive clones were sent for sequencing to verify the mutations and to ensure that no additional alterations had been introduced, as described previously (22).

**Transient expression of HN and F genes.** Transfections were performed according to the PolyFect transfection reagent (Qiagen, Valencia, Calif.) protocol. Briefly, 293T cell monolayers were seeded into T75 culture flasks ($2.4 \times 10^6$ cells/flask) 24 h prior to transfection. Medium was removed from the cell monolayers (40 to 80% confluent) and replaced with 7 ml of fresh 293T cell medium. A transfection mixture containing 8 μg of DNA, 1.3 ml of DMEM, and 80 μl of PolyFect reagent was then added to the culture flask, and the flask was incubated at 37°C for 24 h. The following day, the cells were washed with phosphate-buffered saline (PBS), trypsinized, and lifted from the cell culture flask. Cells were then seeded into 24-well Biocoat plates (Becton Dickinson Labware, Bedford, Mass.) at a density of $5 \times 10^5$ cells/well in 293T cell medium and allowed to grow overnight.

**Assessment of fusion promotion.** HeLa cell monolayers were cotransfected with HN and F constructs as described above, and one of duplicate dishes was supplemented with 4-GU-DANA to a concentration of 2 mM. Thirty hours following transfection and the addition of inhibitor, syncytium formation was assessed microscopically.

**Quantification of cell surface expression of HN by ELISA.** To quantify the amount of HN expressed on the cell surface of 293T cells, an enzyme-linked immunosorbent assay (ELISA) was performed essentially as described previously (1, 3) with the following modifications. Briefly, transfected 293T cells were washed with PBS after incubation at 37°C, fixed for 10 min with 4% formaldehyde in PBS, and reacted with a mixture of anti-HPF3 HN monoclonal antibodies (in PBS supplemented with 0.1% sodium azide and 1% bovine serum albumin [BSA]) supplied by Judy Beeler from the World Health Organization repository. The cells were left at room temperature for 30 min before being washed three times with PBS-BSA. Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Bio-Rad) was then added to the cells in the PBS–1% BSA (1:10,000 dilution), and the cells were incubated for 30 min at room temperature. The cells were then washed three times with PBS-BSA before incubation with the substrate (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid). Absorbance measurements of 50-μl aliquots from each culture well were done at 405 nm on an ELISA reader (Kinetics Reader model EL312e; BIO-TEK Instruments, Winooski, Vt.).

**HAD assays.** Hemadsorption (HAD) was performed and quantitated as described previously (22). Briefly, following aspiration of the medium from transfected 293T cell monolayers in 24-well Biocoat plates (Becton Dickinson Labware), the medium was replaced with 300 μl of 1% red blood cells (RBC) in serum-free medium and the plates were placed at 4°C for 90 min. The wells were then washed three times with 300 μl of cold DMEM (without phenol red). Quantification of the bound RBC was achieved by lysis with 250 μl of RBC lysis solution (0.145 M $NH_4Cl$, 17 mM Tris HCl), and the absorbance was read at 540 nm on an ELISA reader (Kinetics Reader model EL312e; BIO-TEK Instruments).

**RBC release assays.** RBC release assays were performed on 293T cells transiently expressing various constructs as described above. Release assays were performed 48 h following transfection on cells seeded into 24-well Biocoat plates (Becton Dickinson Labware) at a density of $5 \times 10^5$ cells/well. Cell monolayers were washed three times with serum-free medium and placed on a level surface at 4°C with 300 μl of 1% RBC in DMEM for 90 min. The monolayers were then

TABLE 1. Inhibition of HPF3 variant infectivity by 4-GU-DANA[a]

| 4-GU-DANA concn (mM) | % Inhibition[b] of plaques in: | | |
|---|---|---|---|
| | WT | ZM1 | C22 |
| 0.1 | 24.3 ± 14.5 | 0 | 0 |
| 1 | 89.6 ± 1.6 | 0 | 0 |
| 15 | 100 | 26.3 ± 5.7 | 47.7 ± 1.7 |

[a] Plaque reduction assays were carried out as described in Materials and Methods. CV-1 cell monolayers in 24-well plates were infected with WT or variant HPF3 in the absence and presence of 4-GU-DANA.
[b] Means ± standard deviations of results from triplicate culture walls.

rinsed once with cold DMEM (without phenol red) to remove unbound RBC, and 200 μl of DMEM (without phenol red), prewarmed to 37°C, was added to the wells. The plates were then incubated at 37°C for various time periods. At each time point, the plates were rocked to resuspend released RBC and the medium was collected into a V-bottomed 96-well plate. The unreleased (bound or fused) RBC were lysed with 125 μl of RBC lysis solution (0.145 M $NH_4Cl$, 17 mM Tris HCl) and placed in a flat-bottomed 96-well plate. Released RBC in 96-well V-bottomed plates were pelleted at 2,500 rpm for 10 min in a Beckman GS-6R centrifuge with a GH-3.8 rotor equipped with a MicroPlus Carrier. The supernatant fluid was aspirated, and the RBC were lysed in 100 μl of distilled water and transferred to a 96-well flat-bottomed plate. The absorbance values of the released and bound RBC lysis solutions were read at 540 nm (Kinetics Reader model EL312e; BIO-TEK Instruments). The two RBC solutions (released and bound) were then combined, and the absorbance values of the total bound RBC were read at 540 nm.

**Partial removal of sialic acid receptors from RBC.** Partial receptor depletion of RBC was achieved by treatment of 2 ml of a 10% RBC solution in serum-free medium for 2 h at 37°C with 0 to 60 mU of *Clostridium perfringens* NA (type X from *C. perfringens*, catalog no. N-2133; Sigma Scientific). NA was then removed by pelleting the RBC, after which the supernatant fluid was aspirated and replaced with serum-free medium. This washing was repeated three times. Each set of RBC was then resuspended in serum-free medium to make final RBC stocks of 2% RBC.

**NA assays.** NA assays were performed in transiently transfected 293T cell monolayers, as described previously (9, 22).

## RESULTS

**Infectivity of WT and variant viruses in the presence of 4-GU-DANA.** Passage of the WT in the presence of 4-GU-DANA yielded the first variant, ZM1, which showed decreased sensitivity to the plaque-reducing effect of 4-GU-DANA (20). We have now identified a second 4-GU-DANA-resistant variant, C22. This variant was generated in this laboratory by passaging the WT in the presence of exogenously supplied NA (19) and has increased receptor binding avidity. Table 1 shows that 0.1 mM and 1 mM 4-GU-DANA, which reduced WT plaque formation by 24.3 and 89.6%, respectively, had no effect on ZM1 or C22 infectivity; a much higher 4-GU-DANA concentration, 15 mM, reduced the plaque number for these two variants by 26.3 and 47.7%, respectively.

**Cell-cell fusion by WT and variants in the presence of 4-GU-DANA.** To study the effect of 4-GU-DANA on cell-cell fusion separately from that on viral entry, we used cell monolayers that had been cotransfected with WT F and WT or variant HN. The latter included the T193I variant HN, representing one of the two mutations in ZM1 HN. 4-GU-DANA, absent from control wells, was added 2 h after the start of cotransfection. As shown in Fig. 1, all control wells exhibited pronounced cell fusion but only in the case of the WT HN was this fusion completely prevented by 4-GU-DANA. The T193I variant was as resistant to 4-GU-DANA as ZM1, while C22 was somewhat less so. In addition, the I567V variant HN was expressed and showed the same sensitivity to 4-GU-DANA as the WT HN (data not shown). The surface HN expression levels for the WT and each of the HN variants were determined by ELISA (see Materials and Methods), and the WT and variants were expressed at very similar levels from well to well and between variants. The average surface expression of HN variants, presented as a percentage of WT HN expression, was 99.5% ± 13.8% (mean ± standard deviation; minimum difference, 2.2%; maximum difference, 17.9%); F surface expression levels were extremely similar, regardless of the HN variant coexpressed with F, with the average surface expression being 93.4% ± 7.54% (minimum difference, 1.0%; maximum difference, 16.9%) relative to F coexpressed with WT HN.

**NA activity of WT and variants in the presence of 4-GU-DANA.** Our previous studies have shown that even though 4-GU-DANA inhibits HPF3 NA activity, its negative effect on plaque formation and fusion of infected with uninfected cells comes from direct interference with HN-receptor interaction. Nevertheless, it was of obvious interest to compare the mutant and WT HNs with respect to the sensitivity of their NA to 4-GU-DANA and receptor binding activities.

In the experiments whose results are given in Table 2, the NA activities of cells expressing WT or mutant HNs were assayed in the absence of 4-GU-DANA (control) and in the presence of 5 mM 4-GU-DANA, a concentration higher than that required for virtually complete inhibition of WT NA. It may be seen that the control values for ZM1 HN and for the HN with one of the ZM1 mutations, T1931, were at least six times higher than that for WT HN, whereas for the HN with the other ZM1 mutation, I567V, the value was only slightly higher than that for WT HN. While 4-GU-DANA caused over 90% inhibition of the activity of the WT or the I567V variant HN, in the cases of ZM1 and T193I variant HN, the inhibition was only 50 to 60%. For the other variant, C22, the control value and the percent inhibition by 4-GU-DANA were similar or slightly below those for the WT. It is clear that the increased NA activity of ZM1 and its diminished sensitivity to 4-GU-DANA are attributable to the T193I mutation. To further investigate the question of which residue confers resistance and to determine the effect of adding the high-avidity-conferring H552Q mutation of C22 to the T193I alteration, we expressed an HN containing both the C22 mutation and T193I (termed the C22/T193I variant HN). As anticipated, if indeed the increased NA activity and diminished sensitivity to 4-GU-DANA are attributable to T193I, the results for the C22/T193I variant were virtually identical to those for ZM1. Thus, increased baseline NA activity and resistance to 4-GU-DANA are dual consequences of the T193I mutation. For these experiments, the HN expression levels were determined by ELISA for surface HN, as described in Materials and Methods. The WT and variant HNs were expressed at very similar levels from well to well and between variants.

**Receptor binding by WT and variants.** The different sensitivities of WT and variant HPF3s to 4-GU-DANA by the measures of viral infectivity (Table 1) and fusion of HN- and F-expressing cells (Fig. 1) may be due to differences in the extent to which 4-GU-DANA inhibits the receptor binding activities of the HNs. HAD on cells infected with ZM1, compared to those infected with WT, indeed showed greatly di-

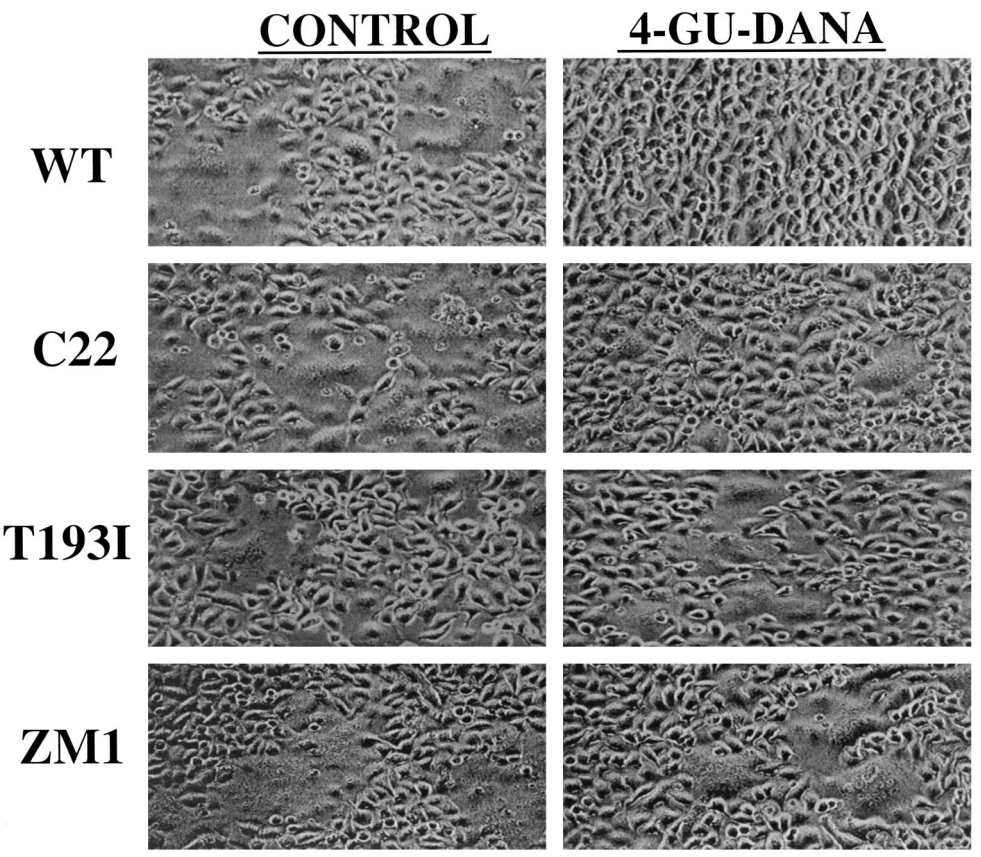


FIG. 1. Effect of 4-GU-DANA on cell-cell fusion in HeLa cell monolayers cotransfected with WT F-expressing and WT or mutant HN-expressing constructs. A 2 mM concentration of 4-GU-DANA was added immediately after cotransfection; photographs of these and of control (lacking 4-GU-DANA) wells were taken 30 h later. Comparable results were obtained with 1 mM 4-GU-DANA.

minished sensitivity to 4-GU-DANA (20). In order to compare the 4-GU-DANA sensitivity of the receptor binding activities of the different HNs more directly and in the absence of other viral molecules, we then used HN-expressing cells. This approach, applicable also to site-directed mutant constructs, permitted the identification of amino acid changes that affect the sensitivity to 4-GU-DANA's inhibition of binding.

TABLE 2. HN neuraminidase activity and its sensitivity to 4-GU-DANA[a]

| Expressed HN | Neuraminidase activity (nmol/min) | |
|---|---|---|
| | Control | 4-GU-DANA |
| WT | 1.37 ± 0.02 | 0.10 ± 0.04 |
| Variants | | |
| C22 | 1.10 ± 0.06 | 0.21 ± 0.08 |
| ZM1 | 9.46 ± 0.73 | 3.51 ± 0.37 |
| T193I | 8.10 ± 1.01 | 4.00 ± 0.39 |
| C22/T193I | 8.19 ± 0.62 | 3.62 ± 0.02 |
| I567V | 2.21 ± 0.16 | 0.11 ± 0.19 |

[a] Cells transfected with the indicated HNs were seeded into 24-well plates. On the next day, the fluorimetric assay of cell surface HN neuraminidase activity (see Materials and Methods) was carried out in the absence or presence of 4-GU-DANA. The cell surface expression of each variant HN was quantitatively compared with that of WT HN; multiplication of these ratios with the neuraminidase activity found for the respective variant HNs yielded the normalized values shown. All values are the means ± standard deviations of results from triplicate wells.

In the experiments whose results are shown in Fig. 2, receptor binding activity was assessed by quantifying HAD (at 4°C) on monolayers of HN-expressing cells in the presence of 0 to 15 mM 4-GU-DANA. For every mutant, HAD at the indicated 4-GU-DANA concentration was compared with that in the absence of 4-GU-DANA and is expressed as the percent inhibition of binding. It can be seen that 0.5 mM 4-GU-DANA completely inhibited RBC binding to WT HN-expressing cells, while 15 mM 4-GU-DANA caused only about 30% inhibition of HAD on ZM1 HN-expressing cells. Between these two extremes are C22 HN-expressing cells, which required about 2 mM 4-GU-DANA for 50% inhibition and more than 5 mM 4-GU-DANA for 80 to 90% inhibition. Figure 2 also shows that the sensitivity of WT HN is unchanged by the introduction of I567V, one of the mutations found in ZM1. In contrast, cells expressing the other mutation in ZM1, T193I, showed a level of resistance to 4-GU-DANA similar to that of ZM1. The role of T193I in 4-GU-DANA resistance is also apparent from results with the C22/T193I variant; it can be seen that the already pronounced resistance of C22 (which required about 20 times more 4-GU-DANA than the WT did for 90% inhibition) was increased by this additional mutation to the extent

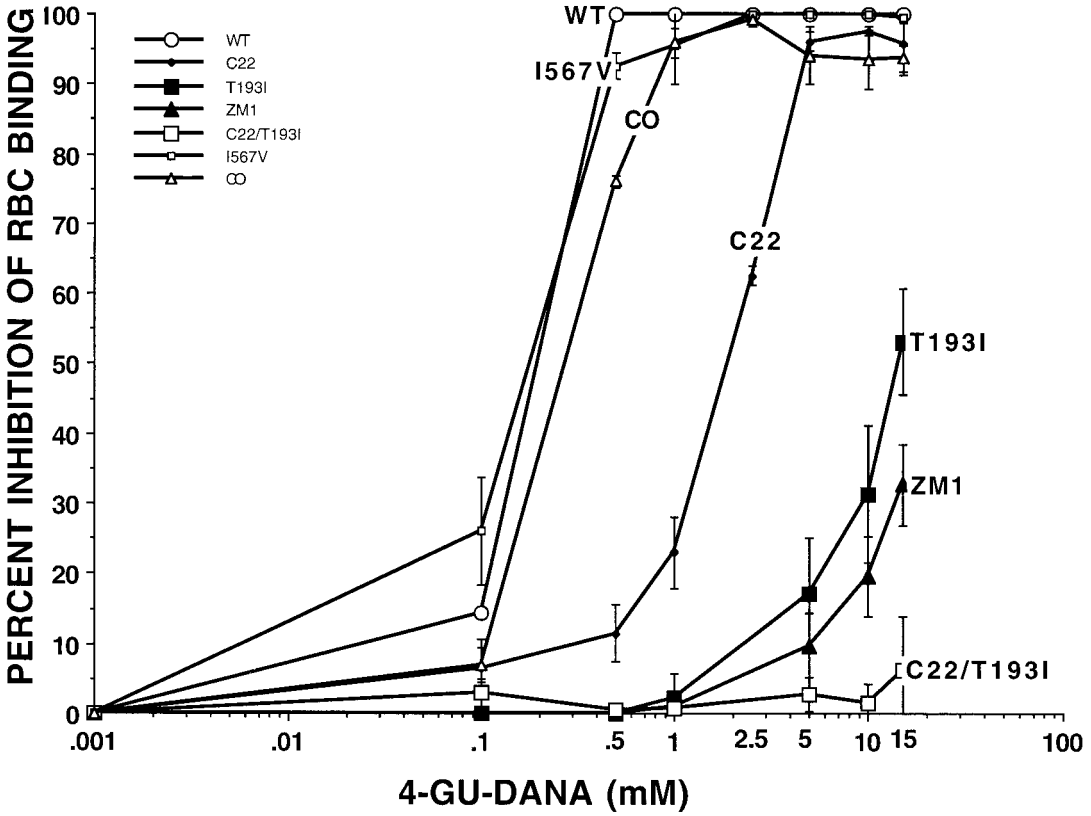


FIG. 2. Sensitivity of HN receptor binding activity to 4-GU-DANA. HAD on cells expressing the WT and the ZM1, T193I, I567V, C22, C0, and C22/T193I mutant HNs was determined in the absence and presence of 4-GU-DANA. The assay conditions and quantification of bound RBC were as described in Materials and Methods. The points (marked by symbols) for percent inhibition (ordinate) at the indicated 4-GU-DANA concentrations (abscissa) are means (bars denote standard deviations) of results on three culture wells.

that even 15 mM 4-GU-DANA failed to significantly inhibit HAD.

**Use of receptor-depleted RBC to assess receptor binding avidity.** In the absence of 4-GU-DANA, the extents of HAD on WT and variant HN-expressing cells were similar. In view of the abundance of receptors on RBC, one may indeed expect that HNs with relatively low receptor binding affinity can adsorb as many RBC as HNs with higher affinity. For this reason, the test system may not be sensitive enough to detect minor differences in receptor avidity. We postulated that such altered avidity can be revealed by the use of RBC partially depleted of cell surface receptors (5, 16, 24) and that the relationship between the degree of this depletion and the extent of HAD on the different HN-expressing cells would give a relative measure of receptor binding avidity. This modification would increase the sensitivity of the assay to differences in binding avidity. RBC were, therefore, treated with different amounts of bacterial NA and then used to quantify HAD on cells expressing different HNs.

Figure 3 illustrates the effect of RBC depletion, expressed as units of NA treatment used for depletion, on HAD, expressed as the percentage of HAD obtained with untreated, nondepleted RBC. (It should be noted that RBC receptor depletion is unlikely to increase linearly with the amount of NA used.) As shown in Fig. 3, the curve for HAD on WT HN-expressing cells undergoes the steepest decline, with RBC binding becoming negligible at a relatively low level of RBC depletion (i.e., 20 mU of NA). At this same level of depletion, HAD on ZM1 or T193I variant HN-expressing cells is still maximal and becomes minimal only at 45 mU of NA. The even slower decline of the curve for the C22/T193I variant HN-expressing cells than that for the T193I variant HN-expressing cells is in harmony with the significant HAD (75%) that C22 itself (unlike the WT) still shows at 20 mU of NA. At this point, HAD also appears to remain significant on cells that express C0 HN (65% versus less than 10% for the WT). C0 variant HN has one mutation, at residue 193; however, the change is from threonine to alanine rather than to isoleucine, as in the case of ZM1. The I567V mutation appears to confer a marginally increased receptor binding capacity, and while this increase is insufficient to result in significant resistance to 4-GU-DANA (see Fig. 2), a slight 4-GU-DANA resistance is apparent when low concentrations of 4-GU-DANA and partially receptor-depleted RBC are used (see Fig. 5 below). The results indicate that increased receptor binding avidity is conveyed by T193I, one of the two mutations in ZM1, and, to a lesser extent, by the single H552Q mutation in C22 and the single T193A mutation in C0.

**Release of RBC from WT and variant HN-expressing cells.** RBC adsorbed on HPF3-infected cells at 4°C can be released at 37°C as a result of receptor cleavage caused by the cells' NA activity. HN-expressing cells were now used to examine the effect of the HN alterations on this process. Since none of the

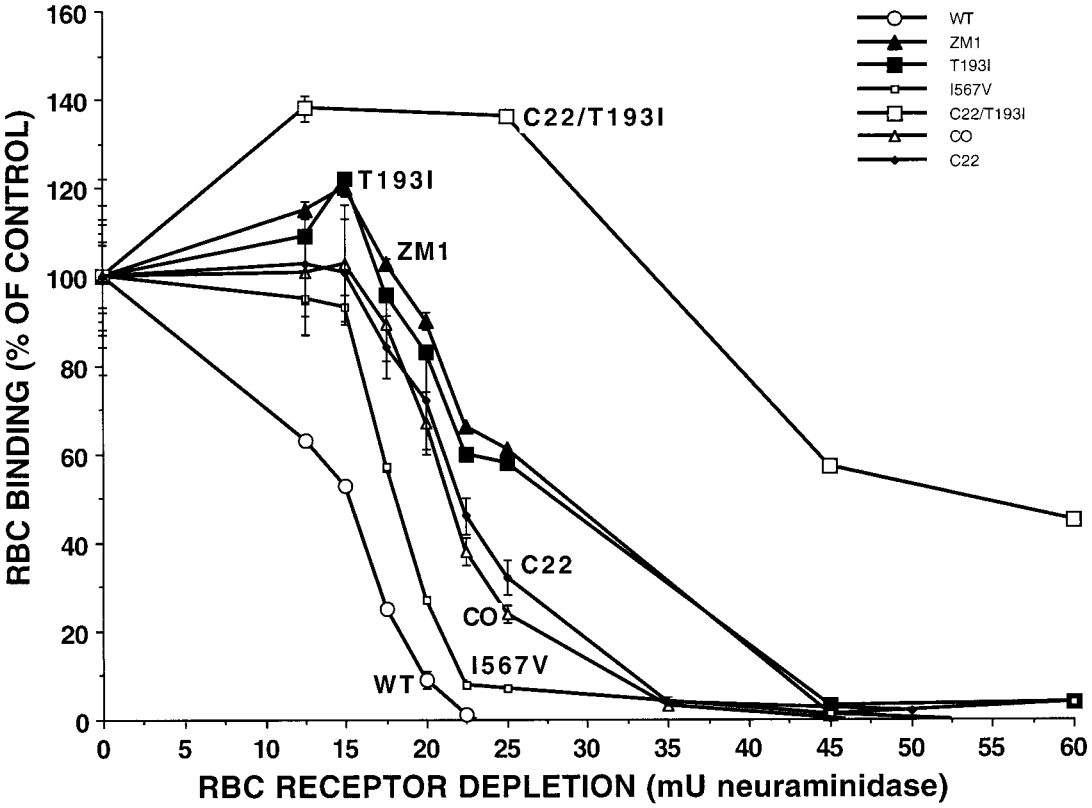


FIG. 3. Relative receptor binding avidity of WT and variant HNs. RBC with different degrees of receptor depletion were prepared by treatment with various amounts of bacterial NA (abscissa) as described in Materials and Methods. Aliquots of these and control (undepleted) RBC preparations were used to quantify HAD on cell monolayers transfected with constructs expressing the WT and the ZM1, T193I, I567V, C22, C0, and C22/T193I variant HNs. After normalization to correct for differences in surface HN expression (Table 2), the extent of binding of each of the depleted RBC is expressed (ordinate) as a percentage of that of the control (i.e., of the amount of untreated, nondepleted RBC bound on cells expressing the corresponding HN). The points are means of results on triplicate monolayers, with bars denoting standard deviations.

mutants studied here were NA deficient, all had the capacity for RBC release; however, the rate of this release over a 2-h period was expected to reveal mutation-associated differences in receptor avidity or NA activity.

The results of comparing the rates of release of RBC receptors at 37°C from WT and mutant HN-expressing cells are shown in Fig. 4. It can be seen that the release of RBC receptors from WT HN-expressing cells continues throughout the experimental period, though at a gradually declining rate. The cells expressing I567V variant HN show a rate of receptor release similar to that of the cells expressing WT HN. For C22 variant HN-expressing cells, there is appreciable release during the first 15 min but none occurs thereafter. This retention must be due to the increased receptor binding avidity of C22 variant HN, since this variant HN has WT NA activity. ZM1 and T193I variant HN also possess increased receptor binding avidity; however, the curves are very similar to that of the WT, suggesting that the manifestation of the increased avidity is precluded by the increased NA activity of these variants. The cells expressing the doubly mutated C22/T193I variant HN show the slowest release of RBC receptors, avidly retaining the RBC. This suggests that the additive effect of the alterations conferring high avidity for the receptor (Fig. 4) result in a highly avid binding that overcomes the T193I variant HN′s increased NA activity.

**Use of depleted RBC to assess effect of 4-GU-DANA on receptor binding of WT and variant HNs.** The lower 4-GU-DANA concentrations required to inhibit HAD on WT HN-expressing cells than that on ZM1 or C22 HN cells (Fig. 3) is consistent with the hypothesis that resistance to 4-GU-DANA is a consequence of increased receptor binding avidity. However, despite the apparently increased avidity of C0 HN for the receptor, we could not detect 4-GU-DANA resistance in this mutant HN by using available methods. We postulated that such resistance might be revealed by the use of those RBC partially depleted of surface receptors (5, 24). In the experiments whose results are shown in Fig. 5, the degree of receptor depletion on the RBC used is defined as for Fig. 3 (expressed as milliunits of NA used for depletion) and HAD was determined as described for Fig. 2. The graph shows that adsorption of nondepleted RBC (0 mU of NA) on C0 HN-expressing cells, as on WT HN-expressing cells, is unaffected by the very low (0.1 mM) 4-GU-DANA concentration. However, the 4-GU-DANA resistance of C0 HN-expressing cells is revealed at both 5.0 and 7.5 mU of NA used for RBC depletion, in that the levels of inhibition of HAD were significantly less (35 and 71%,

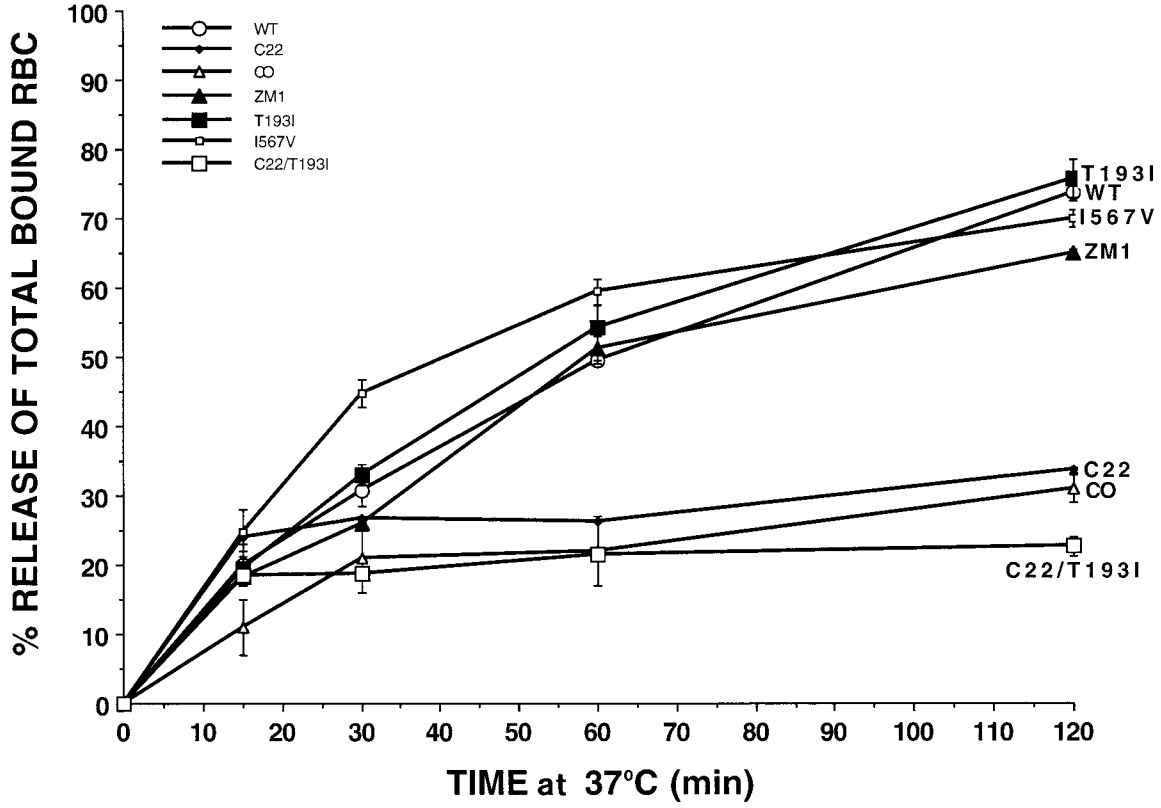


FIG. 4. Release of RBC at 37°C. The rate of release of RBC that had been bound at 4°C to cell monolayers expressing the WT and the ZM1, T193I, I567V, C22, C0, and C22/T193I variant HNs was determined at 37°C. The procedures, detailed in Materials and Methods, include quantification of released RBC as well as of RBC that remained bound at the indicated times after transfer to 37°C. The ordinate values represent released RBC as the percentage of total bound RBC. Each value is a mean ± standard deviation of results for three culture wells. Normalization for differences in the HN surface expression level was as described in Table 2, footnote *a*.

respectively) than those for WT (88 and 101%). As expected from the results shown in Fig. 2, both C22 and T193I variant HN-expressing cells exhibited markedly decreased sensitivity to 4-GU-DANA (compared to that of the WT) with depleted RBC. Furthermore, at a depletion resulting from 10 mU of NA, the percent inhibition of HAD on T193I variant HN-expressing cells was less (17%) than that on C22 HN-expressing cells (30%), which is in accord with the more pronounced resistance of the T193I variant HN than that of the C22 variant HN determined under the conditions described for Fig. 2. Finally, with the use of this low concentration of 4-GU-DANA and the partially receptor-depleted RBC, the slight 4-GU-DANA resistance conferred by I567V is apparent. Thus, the use of receptor-depleted RBC in the experiments whose results are shown in Fig. 5 revealed the 4-GU-DANA resistance of C0 HN and indicates that these variants would be ranked in the order of C0, C22, and variant T193I with respect to both 4-GU-DANA resistance and receptor binding avidity (Fig. 3).

## DISCUSSION

The initial characterization of the newly isolated ZM1 variant revealed that it harbors two HN mutations (20). One of the mutations, T193I, is located at the same residue as the single HN mutation, T193A, in the C0 variant. Since C0 failed to show the strong 4-GU-DANA resistance characteristics of ZM1 (20), it seemed that these differences might be due to either the specific substitution at position 193 or to the I567V mutation in ZM1 HN. This latter possibility was now disproved by the use of singly mutated HNs: we obtained conclusive evidence that the deviations (from WT HN) in all the examined properties of ZM1 variant HN are due to T193I.

The structural similarities between influenza virus NA and paramyxovirus HN suggest that homologous residues might serve similar functional roles. The HPF3 residue T193, which is altered in our ZM1 and C0 variants (to an I and an A, respectively), corresponds to residue E119 in the active-site pocket of influenza virus NA (25) and to NDV HN residue I175 (2). Crystallographic studies of NDV HN indicate that, as in influenza virus NA, the residue at this position lies within the sialic acid binding pocket (3, 4). Substituting residues at I175 in NDV was shown to diminish NA activity, to prevent receptor binding in HAD assays, and to affect fusion promotion ability, clearly indicating the functional importance of this residue for the protein (3, 12, 13, 23).

Due to the structural parallels with influenza virus NA and NDV HN, it seemed likely that HPF3 HN residue T193 provides important interactions with sialic acid receptors and that a mutation at this site could confer resistance to 4-GU-DANA. It was somewhat surprising that the C0 T193A alteration in

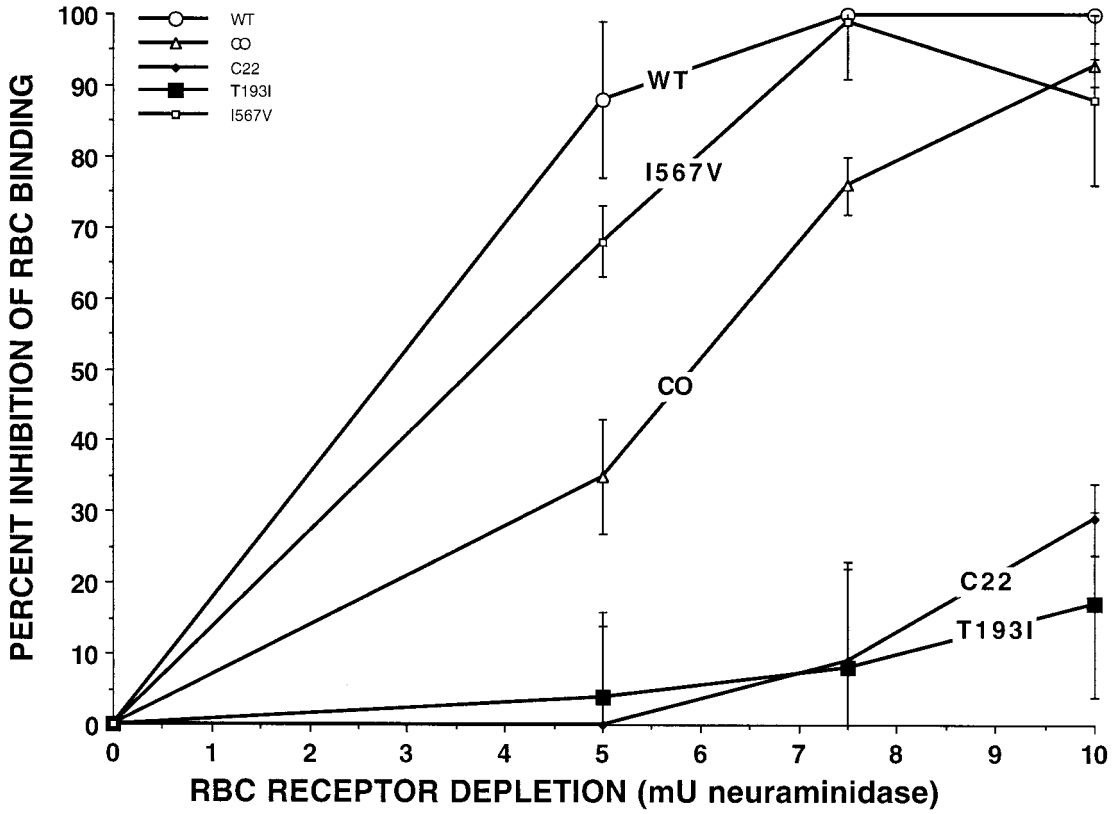


FIG. 5. Effect of 4-GU-DANA on the adsorption of receptor-depleted RBC. HAD on cell monolayers expressing the indicated HN was determined in the presence and absence of 0.1 mM 4-GU-DANA by using RBC with different degrees of surface receptor depletion (see Materials and Methods and Fig. 3). Each value represents the percent inhibition by 4-GU-DANA as a mean ± standard deviation of results for three culture wells.

HPF3 HN did not seem to confer 4-GU-DANA resistance in terms of infectivity or HAD on C0-infected cells (20). We therefore reexamined this question by using C0 HN-expressing cells and the more sensitive assays for HN-receptor interaction developed in the present study. The results showed that T193A did confer significant 4-GU-DANA resistance to the C0 variant HN relative to the WT HN, further emphasizing the importance of residue 193. In addition, the less pronounced 4-GU-DANA resistance of C0 HN (T193A) relative to ZM1 HN (T193I) indicates that the degree of functional alterations depends strongly on whether the original amino acid at this site was replaced by A or I.

ZM1 is the first HPF3 variant found to exhibit increased NA activity. The association of this increase with a rise in the receptor binding avidity of ZM1 HN is in accord with our previous suggestion that a balance between HN′s NA activity and its receptor binding activity is critical for HPF3 propagation (22). A manifestation of such a balance can be seen in experiments on the release of bound RBC from HN-expressing cells at 37°C. For cells expressing ZM1 variant HN, with its increased receptor binding activity as well as NA activity, RBC release was as fast as it was for WT HN-expressing cells (Fig. 4). In variants C22 and C0, studied in parallel with ZM1, no change in NA activity accompanied the elevation of receptor binding avidity (19); consequently, C22 or C0 HN-expressing cells were much less capable of releasing RBC than were ZM1 (or WT) HN-expressing cells, even though the elevation of their receptor binding avidity was less (C22) or much less (C0) pronounced than that of ZM1 HN.

This gradation in the properties of these 4-GU-DANA-resistant variants provided insight into the nature of their resistance. Since C22 and C0 exhibited resistant behavior despite having normal (i.e., WT) NA activity, it seems likely that the 4-GU-DANA resistance of ZM1 is not a result of its increased NA activity. In contrast, the increase in the receptor binding avidity of the variant HNs was directly related to the extent of their 4-GU-DANA resistance, suggesting that this resistance is a consequence of increased receptor binding avidity. Elucidation of the underlying molecular mechanism will require characterization of the site at which 4-GU-DANA binds to HPF3 HN and determination of the affinity of 4-GU-DANA to the different HNs.

In influenza viruses, HA mediates receptor attachment as well as fusion, while the second envelope protein, NA, provides the receptor cleaving activity that permits the release of newly budded virions. In the presence of 4-GU-DANA, which is a powerful selective inhibitor of influenza virus NA (25), these progeny virions remain aggregated on the cell surface, with the consequent curtailment of the spread of infection. In the case of HPF3, on the other hand, 4-GU-DANA hinders infectivity in vitro by interfering with HN-receptor interaction and thus precluding the subsequent steps of fusion and viral entry (6,

22). If infection does occur, the release of progeny HPF3 virions is not hindered by 4-GU-DANA, even though it inhibits the NA activity of HN. Rather, by inhibiting HN-receptor binding, 4-GU-DANA prevents virion aggregation itself, thus obviating the need for receptor cleavage by the NA activity of HN.

The different mechanisms whereby 4-GU-DANA interferes with influenza virus and HPF3 infectivities in vitro suggested that the type of variants arising under the selective pressure of 4-GU-DANA would be different for these two viruses. It has been shown that prolonged growth of influenza viruses in the presence of 4-GU-DANA gives rise to variants with a mutation in the active site of NA but that variants that emerge first are those harboring HA mutations in the receptor binding activity (17). A partial loss of receptor binding ability would diminish the aggregation of progeny virions, and the consequent availability of free virions to infect more cells would counterbalance the effect of 4-GU-DANA, which impedes influenza virus infectivity by preventing the release of progeny virions. Thus, influenza virus escape variants were expected, and found, to include HA mutants with decreased receptor binding avidity. For HPF3, where 4-GU-DANA curtails infectivity by inhibiting HN-receptor interaction, it is reasonable to hypothesize that HN mutants with increased receptor binding avidity are those that can escape 4-GU-DANA's growth inhibitory effect. The present results are in accord with this hypothesis.

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