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Inhibition of Parainfluenza Virus Type 3 and Newcastle Disease Virus Hemagglutinin-Neuraminidase Receptor Binding: Effect of Receptor Avidity and Steric Hindrance at the Inhibitor Binding Sites

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Zanamivir (4-guanidino-Neu5Ac2en [4-GU-DANA]) inhibits not only the neuraminidase activity but also the receptor interaction of the human parainfluenza virus type 3 (HPIV3) hemagglutinin-neuraminidase (HN), blocking receptor binding and subsequent fusion promotion. All activities of the HPIV3 variant ZM1 HN (T193I/I567V) are less sensitive to 4-GU-DANA's effects. The T193I mutation in HN confers both increased receptor binding and increased neuraminidase activity, as well as reduced sensitivities of both activities to 4-GU-DANA inhibition, consistent with a single site on the HN molecule carrying out both catalysis and binding. We now provide evidence that the HPIV3 variant's resistance to receptor-binding inhibition by 4-GU-DANA is related to a reduced affinity of the HN receptor-binding site for this compound as well as to an increase in the avidity of HN for the receptor. Newcastle disease virus (NDV) HN and HPIV3 HN respond differently to inhibition in ways that suggest a fundamental distinction between them. NDV HN-receptor binding is less sensitive than HPIV3 HN-receptor binding to 4-GU-DANA, while its neuraminidase activity is highly sensitive. Both HPIV3 and NDV HNs are sensitive to receptor-binding inhibition by the smaller molecule DANA. However, for NDV HN, some receptor binding cannot be inhibited. These data are consistent with the presence in NDV HN of a second receptor-binding site that is devoid of enzyme activity and has a negligible, if any, affinity for 4-GU-DANA. Avidity for the receptor contributes to resistance by allowing the receptor to compete effectively with inhibitors for interaction with HN, while the further determinant of resistance is the reduced binding of the inhibitor molecule to the binding pocket on HN. Based upon our data and recent three-dimensional structural information on the HPIV3 and NDV HNs, we propose mechanisms for the observed sensitivity and resistance of HN to receptor-binding inhibition and discuss the implications of these mechanisms for the distribution of HN functions.

Attachment of human parainfluenza virus type 3 (HPIV3) to the host cell is mediated by the envelope protein hemagglutinin-neuraminidase (HN). HN binds to sialic-acid-containing receptors on the cell surface and also contributes to the process whereby the other surface protein (the fusion protein F) is triggered and mediates fusion of the viral envelope and the cell membrane. The third role of HN in the infection process is receptor cleavage (via neuraminidase action), allowing for the release of progeny virions and the spread of infection to additional cells (for a review, see reference 9).

One method for interfering with infection by viruses that make use of sialic-acid-containing receptors for entry is the blockade of receptor binding by the use of sialic acid analogs. Monomeric analogs of sialic acid can inhibit the attachment that is required for fusion and entry, and transition-state analogs of sialic acid, identified on the basis of their ability to inhibit influenza neuraminidase, are also effective inhibitors of HPIV3 binding, entry, and fusion (11). 4-guanidino-Neu5Ac2en (4-GU-DANA, or zanamivir) inhibits not only the neuraminidase activity but also the receptor interaction of HPIV3 HN (6), blocking receptor binding and subsequent fusion. For influenza virus, in which 4-GU-DANA inhibits the neuraminidase (NA) and interferes with viral replication by preventing the release of newly formed virions, resistance is conferred by mutations which decrease the binding of 4-GU-DANA to the NA and/or by mutations in the hemagglutinin (HA), which decrease the affinity for the cellular receptor (12). In contrast, for HPIV3, 4-GU-DANA reduces infectivity instead by inhibiting HN-receptor interaction, and hence HN mutants with increased receptor-binding avidities are among those that can escape 4-GU-DANA's growth-inhibitory effect. In fact, for all of the HPIV3 wild-type (wt) and HN variant viruses that we have studied, decreased sensitivity correlated with an increased avidity for the receptor (16, 17).

In earlier work, we selected for an HPIV3 HN variant in tissue culture that was less sensitive to 4-GU-DANA's effects on both HN activities. We thereby generated a fusogenic HPIV3 virus variant (called ZM1 in previous publications [16, 17]; HN T193I/I567V) that harbors two HN gene mutations that result in amino acid alterations and phenotypic resistance to the effects of 4-GU-DANA on both neuraminidase activity and receptor binding (16, 17). One of these mutations (T193I) is responsible for an increase in receptor binding and in neur-

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TABLE 1. Features of HPIV3 HN variants [6]

HPIV3 HN variant	HN mutation(s)	Relative avidity (mU of NA)[a]	NA act (nmol/min)[b]	NA sensitivity to 4-GU-DANA (IC_{50} [mM])	HAD sensitivity to 4-GU-DANA (standard RBC) (IC_{50} [mM])	HAD sensitivity to 4-GU-DANA (receptor-depleted RBC) (IC_{50} [mM])[c]
wt	None	20	1.37 ± 0.02	0.25	0.1	0.02
ZM1 (exists as a virus)	T1931, I567V	37	9.46 ± 0.73	5.0	>20	ND
T1931	T1931	37	8.10 ± 1.01	5.0	>20	0.9
C-22 (exists as a virus)	H552Q	25	1.10 ± 0.06	0.25	2	0.04
T193I/H552Q	H552Q, T193I	50	8.19 ± 0.62	5.0	≫20	0.85

[a] Amount of exogenous neuraminidase required to deplete RBCs so that HAD yields 50% of control binding, normalized to cell surface expression.
[b] Normalized to cell surface HN expression. The cell surface expression of each variant HN was quantitatively compared with that of wt HN. Multiplication of these ratios with the neuraminidase activities found for the respective variant HNs yielded the normalized values shown. All values are means ± standard deviations of results from at least triplicate wells.
[c] The IC_{50} values listed here correspond to those shown in Fig. 1. ND, not done.

aminidase activity as well as for diminished sensitivities of both activities to the inhibitory effect of 4-GU-DANA. An increased receptor-binding avidity accounts for part of the ZM1 (T193I/I567V) variant's 4-GU-DANA-resistant properties. A high-avidity HPIV3 HN variant (H552Q), called C22 in previous publications (15, 17), also exhibits a reduced (intermediate) sensitivity to 4-GU-DANA in terms of receptor binding and infectivity, but without concomitant changes in HN′s neuraminidase activity.

We have shown that an increased receptor-binding avidity is conveyed by T193I (one of the mutations in the zanamivir-resistant T193I/I567V variant) and, to a lesser extent, by the single H552Q mutation in the C22 high-avidity variant. In terms of both receptor-binding avidity and receptor-binding resistance to 4-GU-DANA inhibition, T193I HN ranks higher than H552Q (C22) HN, consistent with the hypothesis that receptor-binding avidity contributes to resistance. However, in terms of the inhibition of neuraminidase activity, several of these variants are sensitive to 4-GU-DANA (H552Q HN and wt HN), while others are less sensitive (T193I/H552Q HN, T193I HN, and T193I/I567V [ZM1] HN). The difference in the order of sensitivity to receptor-binding inhibition versus enzyme inhibition suggests the possibility that 4-GU-DANA's interaction with the binding pocket is less efficient in the neuraminidase inhibition-resistant variants due to the common mutation at T193. Table 1 provides a summary of the sequence alterations and properties of these variant HN molecules and also indicates which of these variant HNs exist in infectious viruses.

The T193 position has been well established in several paramyxoviruses as an important residue for receptor binding and catalysis (3, 4, 15, 17). Only HPIV3 HN has a threonine at this position; the other paramyxovirus HNs contain isoleucine or leucine at this position (16). In the zanamivir-resistant T193I/I567V variant HN, the T193I alteration leads to a sequence at that site which is identical to that of both Newcastle disease virus (NDV) HN and simian virus 5 HN. This raises the possibility that, if I193 in HPIV3 HN confers decreased sensitivity to 4-GU-DANA, the NDV and simian virus 5 HNs may be less sensitive to the effects of this binding inhibitor via mechanisms that are similar to that of the T193I/I567V HPIV3 HN variant. Therefore, we have now tested the hypothesis that in addition to increased receptor avidity, a decreased affinity of the variant HPIV3's receptor-binding site for 4-GU-DANA also contributes to decreased sensitivity to this compound. Our experimental data are placed in the context of recent three-dimensional structural information for HPIV3 HN (10) and of recent structural evidence for a second receptor-binding site on NDV HN (25). We suggest that paramyxovirus resistance to receptor-binding inhibition is related to the ability of each HN to reduce the interaction of these inhibitors with the receptor-binding site and also to the receptor-binding avidity of the particular HN.

MATERIALS AND METHODS

Cells and virus. 293T (human kidney epithelial) cells were grown in Dulbecco's modified Eagle's medium (Mediatech Cellgro) supplemented with 10% fetal bovine serum and antibiotics. For assays of cells in air at different pHs, the medium described above was replaced with a CO_2-independent medium (Invitrogen) which was adjusted according to the manufacturer's instructions to the indicated pHs (21). For NDV infections, a recombinant green fluorescent protein (GFP)-expressing NDV B1 virus (20) was obtained from Peter Palese. For quantitation of the effects of 4-GU-DANA and DANA on viral entry, CHO cell monolayers grown in 1-cm^2 plastic dishes were incubated for 90 min with 5×10^3 PFU of GFP-expressing NDV B1 virus in medium containing various concentrations of inhibitors. The dishes were incubated at 37°C for 24 h, and fluorescent cells in the control and experimental wells were counted under a fluorescence microscope (Olympus 1X70) and photographed with a Sony DKC 500 camera. For quantitation of the effects of 4-GU-DANA on viral growth, supernatant fluids from infected or mock-infected cells containing various concentrations of 4-GU-DANA were collected, and 4-GU-DANA was removed from these fluids by the use of Ultrafree-CL filters (Millipore) according to the manufacturer's instructions. The supernatant fluid was then serially diluted in serum-free medium, and 200 μl of each dilution per well was added to confluent Vero cell monolayers in 48-well plates in the presence of trypsin (30 μg/ml). The cells were incubated at 37°C with intermittent rocking. After 90 min, the inoculum was removed, the medium was replaced, and incubation was continued for 24 h at 37°C. Fluorescent cells were counted as described above.

Chemicals. 4-GU-DANA was prepared from Relenza Rotadisks (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. DANA was obtained from Sigma Chemical Company (St. Louis, Mo.).

HN and F constructs. Mutagenized or wt HPIV3 HN and F cDNAs were digested with either EcoRI and BamHI or SacI and BamHI and then ligated into a digested pEGFP-C3 or pCAGGS mammalian expression vector as previously described (17). NDV B1 Hitchner wt HN was obtained in pCAGGS from Peter Palese.

Transient expression of HN and F genes. Transfections were performed according to the PolyFect transfection reagent protocol (Qiagen, Valencia, Calif.) as previously described (17). Briefly, 293T cell monolayers were seeded into T75 culture flasks (2.4×10^6 cells/flask) 24 h prior to transfection. The 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 Dulbecco's modified Eagle's medium, and 80 μl of PolyFect reagent was

then added to the culture flask and incubated at 37°C for 24 h. The cells were lifted from the cell culture flask on the following day. The cells were then seeded into 24-well Biocoat plates (Becton Dickinson Labware, Bedford, Mass.) at a density of 5×10^5 cells/well in 293T medium and allowed to grow overnight.

Quantification of cell surface expression of HN by ELISA. To quantify the amount of HN expressed on the cell surfaces of 293T cells, we performed an enzyme-linked immunosorbent assay (ELISA) as described previously (17). Transfected 293T cells were washed with phosphate-buffered saline (PBS) after incubation at 37°C, fixed for 10 min with 4% formaldehyde in PBS, and incubated with a mixture of anti-HPF3 HN monoclonal antibodies supplied by Judy Beeler from the World Health Organization repository (in PBS supplemented with 0.1 M sodium azide and 1% bovine serum albumin [BSA]) or anti-NDV B1 HN monoclonal antibodies supplied by Thomas Moran. The cells were left at room temperature for 30 min and then washed three times with PBS-BSA. Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Bio-Rad) was then added to the cells in PBS–1% BSA (1:10,000 dilution), and the cells were incubated for 30 min at room temperature. The cells were washed three times with PBS before incubation with substrate (2,2′-azino-bis; 3-ethylbenzthiazoline-6-sulfonic acid). Absorbance measurements were taken at 405 nm with an ELISA reader (Power Wave X equipped with KC4 Kineticalc for Windows v. 2.7; Bio-Tek Instruments, Winooski, Vt.).

HAD assays. Hemadsorption (HAD) assays were performed and quantified as previously described (22). After 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 (RBCs) in serum-free, CO_2-independent medium (at the indicated pH), and the cells were incubated at 4°C for 30 min. The wells were then washed three times with 300 μl of cold CO_2-independent medium (Gibco, Gaithersburg, Md.). The bound RBCs were lysed 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 (Power Wave X equipped with KC4 Kineticalc for Windows v. 2.7; Bio-Tek Instruments).

Partial removal of sialic acid receptors from RBCs. Partial receptor depletion of RBCs 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 100 mU of *Clostridium perfringens* NA (type X; Sigma) as described previously (17). NA was then removed by pelleting the RBCs, after which the supernatant fluid was aspirated and replaced with serum-free medium. This washing process was repeated three times. Each set of RBCs was then resuspended in serum-free, CO_2-independent medium to make final RBC stocks of 2% RBCs.

Use of receptor-depleted RBCs to assess HPIV3 HN receptor-binding avidity. RBCs that were partially depleted of their surface sialic acid receptors (described above) were used to determine the relative receptor binding avidities of variant HN molecules. For each experiment, all of the human RBCs were from the same preparation of depleted pools. In experiments that included avian RBCs for comparison, the human RBCs were from one pool and the avian RBCs were prepared separately. Note that there was a high degree of reproducibility between preparations, but whenever possible we performed experiments using single preparations to simplify the consistency of comparisons. The RBCs were overlaid onto 48-well plates of 293T cell monolayers that had been transiently transfected 48 h prior with wt or variant HN as described above. The plates were placed at 4°C for 30 min to allow RBC binding. The cell monolayers were then washed at 4°C with cold CO_2-independent medium to remove unbound RBCs, the bound RBCs were lysed (0.145 M NH_4Cl, 17 mM Tris-HCl), and the absorbance was read at 540 nm on an ELISA reader (Power Wave X equipped with KC4 Kineticalc for Windows v. 2.7; Bio-Tek Instruments). The results are presented as percent retention of RBCs relative to control (undepleted RBCs) versus degree of depletion in milliunits of bacterial neuraminidase.

Neuraminidase assays. Neuraminidase assays were performed with transiently transfected 293T cell monolayers as previously described (17).

RESULTS

Decreased drug binding to HPIV3 HN contributes to the T193I mutant's decreased sensitivity to 4-GU-DANA. In order to assess whether reduced affinity of the variant HN′s receptor-binding pocket for 4-GU-DANA contributes to its decreased sensitivity to this compound, we developed a HAD-based assay for binding inhibition that allowed us to determine the affinity for 4-GU-DANA which was independent of the receptor-binding avidity. The assay was based on a modification of an earlier assay that we used for quantitative comparisons of binding avidities of HNs, by which we found that we could detect small differences in avidity by measuring binding to RBCs that were partially depleted of cell surface receptors by bacterial neuraminidase (17, 23). We determined the amount of neuraminidase treatment that reduced binding to 50% for each variant. Treating RBCs so that the binding of each variant was reduced minimized binding avidity differences between variants. High-avidity HNs required more depletion of RBC receptors than wt HNs. We then assessed the ability of different 4-GU-DANA concentrations to compete for RBC binding in HAD assays by using the depleted RBCs for each variant. These depleted RBC pools were allowed to bind to cells that were transfected with the wt and variant HN molecules. Each experiment was performed with a single RBC preparation to enhance the consistency of comparisons. This strategy allowed the variants to compete under reproducible conditions and permitted an isolated assessment of affinities for 4-GU-DANA.

Figure 1 shows the amounts of 4-GU-DANA that were required to inhibit the binding of wt HN, H552Q HN, T193I HN, and T193I/H552Q HN to their 50% depleted RBCs. Table 1 shows that the variants ranked in terms of receptor avidity in the order wt HN < H552Q HN < T193I HN < T193I/H552Q HN and that the 50% inhibitory concentrations (IC_{50}s) for binding of these variants to untreated RBCs were different. However, the results in Fig. 1 show that when the 50% depleted RBCs were used (instead of untreated RBCs), the variant HNs clustered in only two 4-GU-DANA groups: wt HN and H552Q HN grouped together, and T193I HN and T193I/H552Q HN grouped together. The curves for the wt and H552Q HNs are virtually identical, and the curves for the T193I and T193I/H552Q HNs are virtually identical. Since under these conditions the wt and variant HNs all bind with the same net receptor avidity, the differences that we detected in the ability of 4-GU-DANA to compete with this binding were likely due to differences in the affinities for 4-GU-DANA itself. These results thus support the hypothesis that the T193I alteration may confer a decreased sensitivity to 4-GU-DANA by reducing the affinity of the binding site for 4-GU-DANA and that 4-GU-DANA resistance is due not only to alterations in receptor-binding avidity but also to reduced binding of 4-GU-DANA itself. In contrast, the reduced 4-GU-DANA sensitivity of the H552Q HN appears to be due only to alterations in receptor-binding avidity, since it binds 4-GU-DANA with a similar affinity as the wt HN.

NDV HN receptor-binding inhibition by 4-GU-DANA. The position corresponding to HPIV3 T193 has been established in several paramyxoviruses as an important residue for both receptor binding and catalysis (3, 4, 15, 17). We noted that residues in the immediate vicinity of HPIV3 T193 that contact sialic acid are conserved in NDV HN. Since NDV HN has an isoleucine (I175) at this position and since we have shown that a mutation of threonine to isoleucine has multiple effects on HPIV3 HN, we were interested in determining the ability of 4-GU-DANA to inhibit the receptor-binding activity of the NDV HN molecule. HAD was carried out on monolayers of cells expressing approximately equivalent levels of HPIV3 wt HN, HPIV3 T193I HN, HPIV3 H552Q HN, HPIV3 T193I/H552Q HN, and NDV HN in the presence or absence of 4-GU-DANA. The results (Fig. 2) show that NDV HN has a

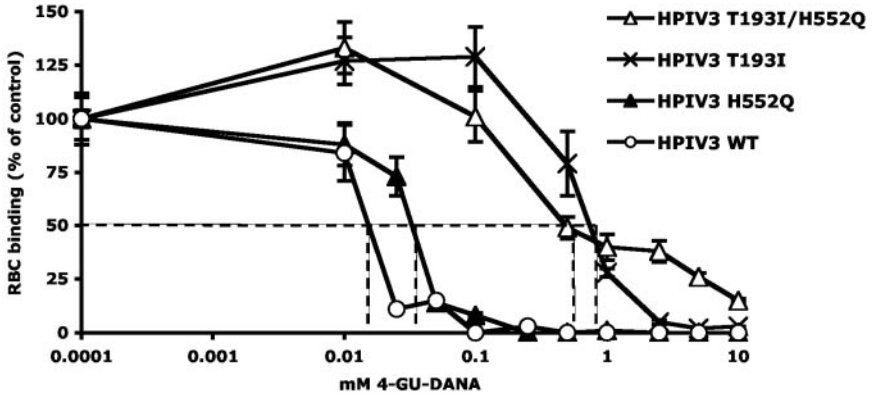

FIG. 1. RBC binding to HN variants in the presence of 4-GU-DANA under conditions controlling for HN receptor-binding avidity. HAD on 293T cell monolayers transiently expressing HPIV3 HNs (wt HN, H552Q HN, T193I HN, and T193I/H552Q HN) was determined in the absence and presence of 4-GU-DANA. RBC pools with different degrees of receptor depletion were prepared by treatments with various amounts of bacterial neuraminidase as described in Materials and Methods and were used to quantify HAD on cell monolayers. The degree of RBC receptor depletion providing 50% of the control (undepleted) binding was determined for each HN variant, and depleted RBC pools were used with their respective HNs to determine the HAD in the presence of various concentrations of 4-GU-DANA (abscissa). The extent of binding of each HN variant's RBC pool (ordinate) is expressed as a percentage of that of the control (the amount of the depleted RBC pool bound to cells expressing the corresponding HN in the absence of 4-GU-DANA). The points are means of results on triplicate cell monolayers from representative experiments repeated between three and seven times, with bars denoting standard deviations. The IC_{50} for each HN is indicated by the dashed line.

strikingly lower sensitivity to the effect of 4-GU-DANA on receptor binding than that of wt HPIV3 HN. The reduced sensitivity is similar to that seen with the HPIV3 HN variants with a T193I mutation.

Assessment of NDV HN receptor-binding avidity. Since an increased receptor avidity can decrease 4-GU-DANA sensitivity in HPIV3, we assessed the receptor-binding avidity of NDV HN in parallel with those of HPIV3 wt HN, T193I HN, H552Q HN, and T193I/H552Q HN. Binding to RBCs was determined after treatments with different concentrations of bacterial neuraminidase, as for the experiment depicted in Fig. 1. The results (Fig. 3) show that even low concentrations of neuraminidase were sufficient to dramatically reduce NDV binding. Hence, NDV HN has low avidity for both avian and human RBCs compared to the HPIV3 HNs. Therefore, in contrast to the case for the HPIV3 T193I variant HNs, the Ile at NDV residue 175 (corresponding to residue 193 in HPIV3) does not confer increased receptor avidity. Increased avidity thus does not account for the decreased sensitivity of NDV HN to 4-GU-DANA seen in Fig. 2. Note that NDV showed similar avidity in this assay for both avian and human RBCs and that the sensitivity to 4-GU-DANA was similar regardless of the RBC type (Fig. 2).

HPIV3 HN and NDV HN receptor-binding inhibition by Neu5Ac2en (DANA). To test the hypothesis that the cause of the reduced interaction of 4-GU-DANA with the receptor-binding pocket in T193I HN and NDV HN is potentially due to unfavorable interactions between the guanidinium group and the respective HN, we determined the sensitivity of these HN molecules to the smaller inhibitor Neu5Ac2en (DANA), which

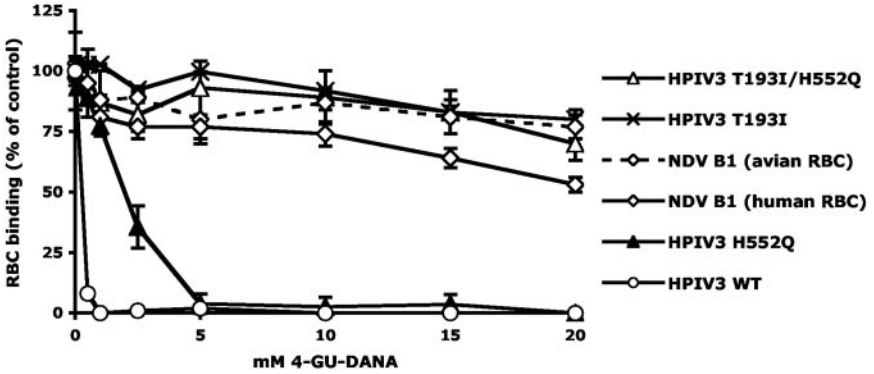

FIG. 2. HN receptor-binding sensitivity to 4-GU-DANA. HAD on 293T cell monolayers transiently expressing HPIV3 HNs (wt HN, T193I HN, H552Q HN, and T193I/H552Q HN) or NDV HN was determined at pH 7.3 in the absence and presence of 4-GU-DANA. NDV HN was assayed using either human RBCs as for HPIV3 (solid line) or avian RBCs (dashed line). The assay conditions and quantification of bound RBCs were described in Materials and Methods. The extents of binding to each HN (ordinate) at the indicated 4-GU-DANA concentrations (abscissa) are expressed as percentages of that of the control (the amount of RBCs bound on cells expressing the corresponding HN in the absence of 4-GU-DANA). The data points are means (bars denote standard deviations) of results on triplicate cell monolayers from representative experiments repeated between three and seven times.

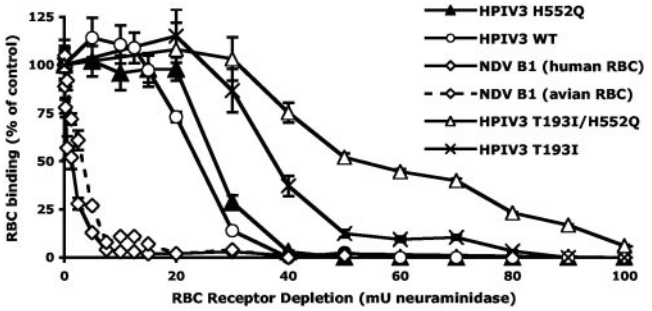

FIG. 3. Relative HN receptor-binding avidities of HPIV3 and NDV HN. RBCs with different degrees of receptor depletion were prepared by treatments with various amounts of bacterial neuraminidase (abscissa) as described in Materials and Methods. Aliquots of these and control (undepleted) RBC preparations were used to quantify HAD on cell monolayers transiently expressing HPIV3 wt HN, HPIV3 T193I HN, HPIV3 H552Q HN, HPIV3 T193I/H552Q HN, or NDV (B1 Hitchner strain) HN. The extent of binding of each of the depleted RBC preparations (ordinate) is expressed as a percentage of that of the control (the amount of untreated, undepleted RBCs bound on cells expressing the corresponding HN). The points are means of results on triplicate cell monolayers from representative experiments repeated between three and seven times, with bars denoting standard deviations.

lacks the guanidinium group. Figure 4 compares the sensitivities to DANA of HPIV3 wt HN, HPIV3 T193I HN, HPIV3 H552 HN, and HPIV3 T193I/H552Q HN with that of NDV B1 HN and shows that all the HNs, both those that are sensitive and those with decreased sensitivities to 4-GU-DANA, were sensitive to inhibition of binding by DANA. For the HPIV3 HNs, as avidity for receptor increased, DANA sensitivity increased. The most striking finding of this experiment was that NDV B1 HN was only partially sensitive to DANA and that it had residual receptor binding (approximately 40%) which

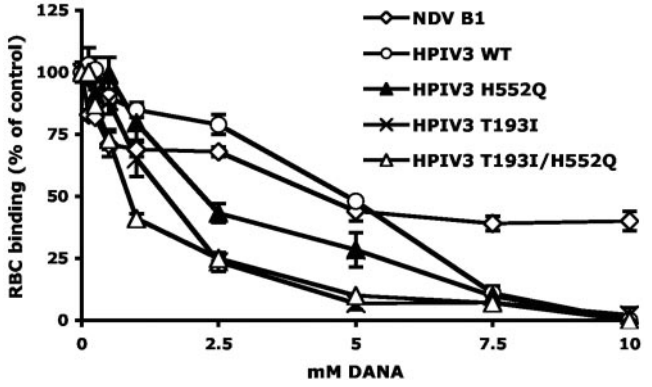

FIG. 4. HN receptor-binding sensitivities to Neu5Ac2en (DANA). HAD on 293T cell monolayers transiently expressing HPIV3 HNs (wt HN, T193I HN, H552Q HN, and T193I/H552Q HN) and NDV HN was determined in the absence and presence of DANA. The assay conditions and quantification of bound RBCs were as described in Materials and Methods. The extents of binding to each HN (ordinate) at the indicated DANA concentrations (abscissa) are expressed as percentages of that of the control (the amount of RBCs bound on cells expressing the corresponding HN in the absence of DANA). The data points are means (bars denote standard deviations) of results on triplicate cell monolayers from representative experiments repeated between three and seven times.

could not be completely inhibited even at the highest concentrations of DANA.

NDV neuraminidase activity is sensitive to both 4-GU-DANA and DANA. We have previously shown that wt HPIV3 neuraminidase activity is inhibited 92% by 5 mM 4-GU-DANA (17), whereas the T193I HN neuraminidase is far less sensitive (17). We therefore tested the neuraminidase sensitivity of NDV HN. We performed neuraminidase inhibition assays with 293T cells expressing NDV HN, using a 10 mM concentration of the substrate MUNANA. In each instance, HN expression levels were determined and the results were normalized (as described in Table 1). The NDV HN was inhibited about 90% in the presence of either 5 mM 4-GU-DANA or 5 mM DANA. The NDV HN neuraminidase activities were 22.8 $\pm$ 2.6 nmol/min for the control, 2.5 $\pm$ 0.7 nmol/min in the presence of 5 mM 4-GU-DANA, and 2.6 $\pm$ 0.4 nmol/min in the presence of 5 mM DANA. NDV neuraminidase inhibitor sensitivity is thus essentially the same as that of wt HPIV3. Thus, NDV HN does not possess a neuraminidase that is resistant to 4-GU-DANA like the T193I HN neuraminidase, even though it possesses an isoleucine at the residue that is homologous to the 193 position in HPIV3 HN.

NDV HN′s decreased sensitivity to the effect of 4-GU-DANA on receptor binding is independent of pH. Since we had demonstrated the sensitivity of the NDV neuraminidase to 4-GU-DANA inhibition at pH 6.5 and since the receptor-binding assays had all been performed at pH 7.3, we questioned whether 4-GU-DANA may bind with a higher affinity at the lower pH. We therefore tested the sensitivity of NDV HN receptor binding to 4-GU-DANA at pH 6.5. HAD on 293T cells that transiently expressed HPIV3 wt and NDV HN was determined in the absence and presence of 4-GU-DANA at pH 6.5, and we found that a decreased sensitivity to binding inhibition persisted at this lower pH (data not shown).

Effects of 4-GU-DANA and DANA on viral growth. Since we found that 4-GU-DANA is an effective inhibitor of NDV neuraminidase, using expressed HN, we were interested in determining whether this degree of neuraminidase inhibition leads to an inhibition of NDV growth in culture by virtue of inhibiting viral release. For quantitation of the effects of 4-GU-DANA on NDV viral growth, plaque assays were used to assess the amount of infectious particles released from the infected cells. Vero cells were infected with NDV B1 in the presence of the inhibitor at various concentrations. Supernatant fluids from infected or mock-infected cells in the presence of various concentrations of 4-GU-DANA were collected 24 and 48 h after infection, cleared of 4-GU-DANA, and allowed to form plaques on monolayers of Vero cells, and the plaques were then counted. Infection in the presence of 10 mM 4-GU-DANA resulted in a 10-fold decrease in the number of plaques (PFU) released from the NDV-infected cells after 24 h of infection, and the IC_{50} of 4-GU-DANA against NDV in cell culture was 1.2 mM. Forty-eight hours after infection, the inhibitory effect was reduced: 1.2 mM 4-GU-DANA resulted in only a 15% reduction in PFU compared to untreated infected cells (data not shown). These results represent the means of results from three different experiments done in triplicate. Since NDV receptor binding is resistant to the inhibitory effects of 4-GU-DANA, this result suggested that the

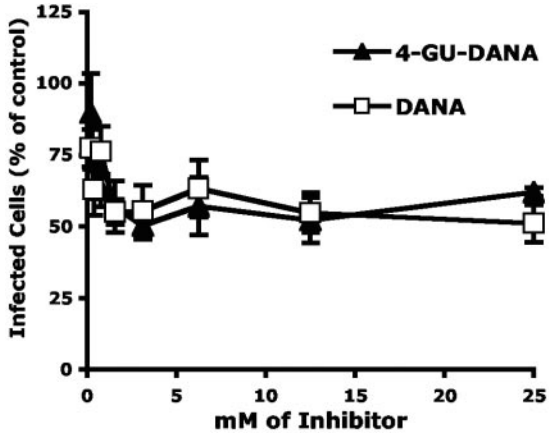

FIG. 5. Effects of 4-GU-DANA and DANA on NDV viral entry. CHO cells in 48-well dishes were infected with 5×10^3 PFU of NDV B1 virions expressing GFP in the presence of the inhibitors at various concentrations. Twenty-four hours after infection, the cells were washed, and fluorescent cells in the control and experimental wells were counted under a fluorescence microscope.

decrease in infectious particles released from infected cells is accounted for by the inhibition of neuraminidase.

Effects of 4-GU-DANA and DANA on viral entry. Figure 4 showed that DANA does not completely block NDV receptor binding; residual receptor binding of approximately 40% could not be completely inhibited even at the highest concentrations of DANA used. It was therefore of interest to ask whether DANA is correspondingly unable to completely inhibit the entry of NDV into cells in culture. We proposed that DANA-resistant receptor binding to the resistant second site can mediate viral entry into cells. Figure 5 shows the effect of DANA and 4-GU-DANA on the viral entry of NDV B1 virions expressing GFP (20). This recombinant NDV possesses an F protein that lacks a cleavage site so that progeny virions cannot infect new target cells unless they are treated with an exogenous protease. The use of this recombinant NDV thus allows for an isolated assessment and quantitation of viral entry, since only the virions in the initial inoculum will infect cells. Viral entry was quantitated by counting fluorescent cells in the control and experimental wells, and the results are expressed in Fig. 5 as the percentages of fluorescent (infected) cells compared to a control infection in the absence of the inhibitor. Even at DANA concentrations of 6.12, 12.5, and 25 mM, significant viral entry was evident, and the extent of this entry did not diminish as the DANA concentration rose above 0.75 mM. For the experiments with 4-GU-DANA, although the data in Fig. 2 showed that receptor binding was even more resistant to 4-GU-DANA than to DANA (approximately 75% residual binding), there was a partial inhibition of entry, but significant entry was retained even at the highest concentrations of the compound. These data show that the binding activity of the NDV HN that is DANA resistant is indeed sufficient to mediate viral entry. Note that the actual rates of binding inhibition in transfected cells with known amounts of HN expression, as shown for NDV in Fig. 2 and 4, were not quantitatively comparable with the rates of plaque appearance of fluorescent infected cells after viral entry, since the latter is a multistep process with variations in HN and F expression during viral entry.

DISCUSSION

To answer questions about the mechanism of 4-GU-DANA (zanamivir) action in HPIV3 infection, we used 4-GU-DANA to select for escape variant viruses in vitro (16). We showed that the different sensitivities of wt and variant HPIV3 HNs to 4-GU-DANA, as measured by the viral infectivity or fusion of HN- and F-expressing cells, are due to differences in the extent to which 4-GU-DANA inhibits the HN′s receptor-binding activity. We found several indications, however, that an enhanced binding avidity did not account entirely for the observed decreased sensitivity to 4-GU-DANA. Variant HNs with a T193I mutation that conferred increased receptor avidities also had altered neuraminidase activities and decreased enzyme sensitivities to 4-GU-DANA, whereas no effect on neuraminidase sensitivity was seen with the high-avidity H552Q mutation. For variant HPIV3 HNs containing a T193I substitution, our experimental data (Fig. 1) demonstrate that in addition to increased avidities for the receptor, decreased affinities for 4-GU-DANA contribute to the decreased sensitivities of these variants to 4-GU-DANA in a receptor-binding assay. This is analogous to influenza virus variants with decreased sensitivities to 4-GU-DANA that have lower affinities for 4-GU-DANA in the enzyme active site (2). The fact that this substitution conferred a decreased sensitivity to 4-GU-DANA but not to DANA (a molecule that is identical to 4-GU-DANA except for a smaller substituent group at C-4) provides further support for the view that decreased binding to 4-GU-DANA, consequent to the substitution of an Ile (but not of Ala) at T193, contributes to decreased sensitivity.

The recently published three-dimensional structures of HPIV3 HN (10), taken together with the suite of NDV HN structures (4, 25), permit a structural interpretation of these results. In the HPIV3 HN structural work (10), it was suggested that the resistance to zanamivir of the HPIV3 HN T193I mutant may be related to the increased size of the side chain at that position. Figure 6 now elaborates in more detail how this might arise. We noted that (i) all atoms common to residues HPIV3 HN T193 and NDV HN I175 have almost identical dispositions within these molecules, (ii) atoms common to the 4-GU-DANA and DANA ligands have almost identical dispositions within their respective active sites, and (iii) the residues surrounding HPIV3 HN T193 (viz R192, N208, V322, and Y530) are conserved in NDV HN (as R174, N190, V302, and Y526, respectively). It thus seems plausible to suggest that a T193I mutation in HPIV3 HN would place the side chain of the isoleucine in the same conformation as that seen for the side chain of I175 in NDV HN. However, such a conformation would indeed lead to an immediate steric clash between the Cδ methyl group of I175 and the guanidinium moiety of 4-GU-DANA if the ligand were bound in the same conformation as that observed for its complex with the wt. In silico modeling showed that this steric clash cannot be relieved simply by allowing the isoleucine side chain to adopt an alternative rotameric (5) conformation, i.e., relaxation of the steric clash with the ligand requires (i) that the isoleucine side chain adopts an unfavorable (i.e., nonrotameric) conformation, (ii) a

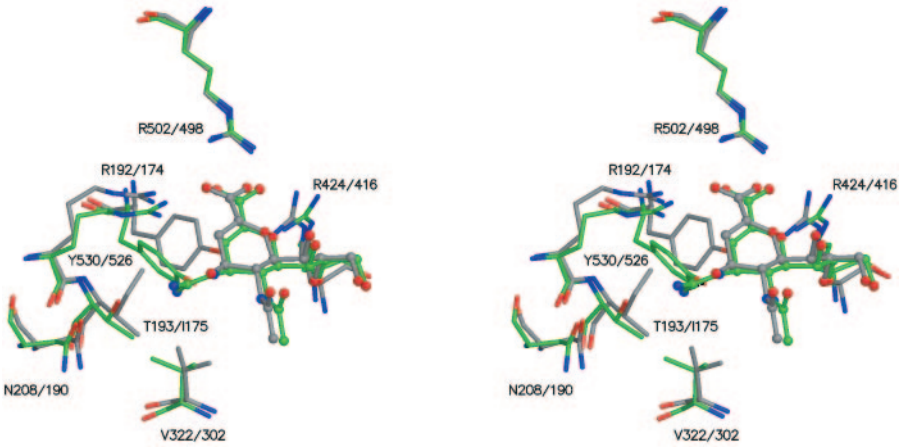

FIG. 6. 4-GU-DANA bound to HPIV3 HN compared to DANA bound to NDV HN. The figure shows (in stereo) selected residues in the vicinity of T193 in HPIV3 in its zanamivir-complexed form (10) overlaid with the equivalent residues (in the vicinity of I175) in NDV HN in its DANA-complexed form at pH 6.5 (4). HPIV3 HN residues are shown in stick representation with bound zanamivir shown in ball-and-stick representation (carbon atoms, green; oxygen atoms, red; nitrogen atoms, blue). The NDV HN residues are also shown in stick representation with bound DANA shown in ball-and-stick representation (carbon atoms, gray; oxygen atoms, red; nitrogen atoms, blue). Individual residues are labeled *Xnnn*/*Ymmm*, where *X* and *Y* are the single-letter codes for the respective HPIV3 and NDV HN residues and *nnn* and *mmm* are their respective residue numbers. The atomic coordinates were extracted from the Protein Data Bank (1) from entries 1V3E and 1E8V, respectively, and were overlaid, using LSQMAN software (7). The figure was generated with MOLSCRIPT (8) and RASTER3D (14).

dispositional movement on the part of the ligand, or (iii) a conformational rearrangement on the part of the surrounding protein side chains. These structural observations, taken together, thus support the hypothesis that the resistance of the T193I HN variant to 4-GU-DANA (Fig. 1) arises from a reduction in the binding of 4-GU-DANA associated with the steric bulk of the I193 side chain.

The effect on neuraminidase activity of the T193I mutation in HPIV3 HN can also be interpreted in the light of the atomic structures. The T193I HPIV3 HN mutant has been shown previously to exhibit increased neuraminidase activity with respect to the wt (17) (Table 1). Accommodation of the additional methyl group within the isoleucine side chain at the same rotameric conformation as that observed for its counterpart, I175, in NDV HN (Fig. 6) could potentially have the effect of reducing the mobility of the catalytically important residue Y530 (4) and hence potentially leading to an increase in the catalytic rate. Such an explanation would also be consistent with the observation that NDV HN (which has an isoleucine at the same position) has a higher enzymatic activity than wild-type HPIV3 HN.

However, the above lines of argument may appear to be at odds with our observation that the neuraminidase activity of NDV HN (which has a wild-type isoleucine at position 175, corresponding to HPIV3 HN T193) is sensitive to 4-GU-DANA. One possible explanation for the difference in sensitivity compared to that of T193I HPIV3 HN is that the residues surrounding NDV HN I175 have more flexibility than their counterparts in HPIV3 HN and thus the isoleucine side chain in NDV HN can adopt conformations that would be energetically unfavorable in T193I HPIV3 HN. Such a suggestion is speculative, however, and the issue can only be resolved by a determination of the structure of NDV HN in complex with 4-GU-DANA.

While the T193I HN had the highest resistance among the variants to 4-GU-DANA (with the effect of receptor avidity factored out) (Fig. 1), under the same conditions the H552Q HN (C22) variant was as sensitive to 4-GU-DANA as the wt, despite its higher receptor avidity. This mutation had no effect on neuraminidase activity or on neuraminidase sensitivity to 4-GU-DANA (Table 1). This result can also be examined in a structural light. H552 lies at the C terminus of the second strand of the sixth β-sheet (β_6S_2) within the HN monomer (Fig. 7). The loop between β_6S_2 and the following strand, β_6S_3, forms part of the dimeric interface both in the HPIV3 HN structure (10) and in the NDV HN structure at pH 6.5 (4). An inspection of the HPIV3 HN structure in complex with DANA (Fig. 6) shows (i) that no atom of this residue is involved in the formation of the ligand-binding site and (ii) that a substituted glutamine side chain may be accommodated at H552 without a prima facie requirement for structural rearrangement within its immediate environment. This structural observation is thus compatible with the mutation having no effect on the 4-GU-DANA binding affinity or on the neuraminidase activity. However, it does not explain the increased receptor-binding avidity of the H552Q variant (15, 17). Possible, though highly speculative, explanations for this observation are (i) that the mutation causes an indirect alteration in the conformation of the binding site, possibly via the side chain of E549 lying within the same strand, or (ii) that the increase in avidity is caused by a change in a putative second receptor-binding site. In the latter scenario, the H552Q mutation would increase the receptor avidity at the second site but would not alter the avidity at the first (4-GU-DANA-sensitive and neuraminidase-active) site.

The issue of whether the neuraminidase activities and receptor-binding activities of paramyxoviral HNs reside on separate sites has been the subject of much investigation. Early studies on NDV indicated that sialic acid analogs (such as DANA and FANA) had different effects on hemagglutination and neuraminidase activity, leading to the suggestion that sep-

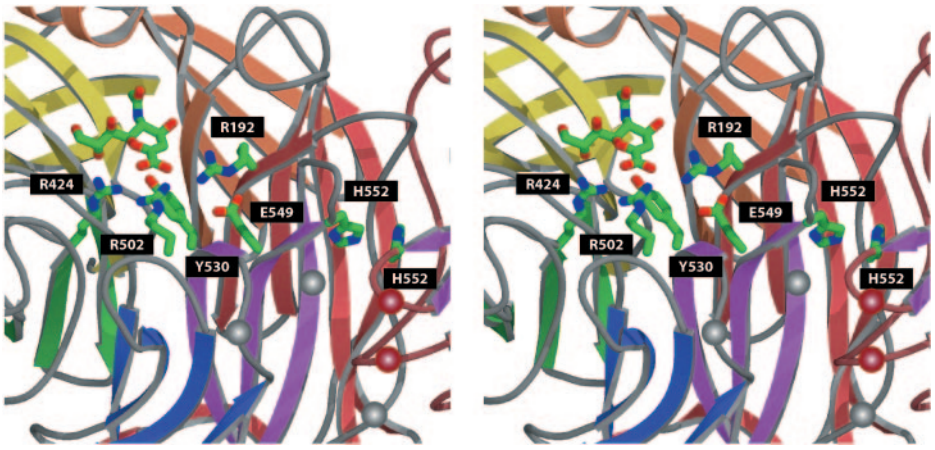

FIG. 7. HPIV3 HN dimer interface in the vicinity of H552. The figure is a stereo schematic view of part of the dimer interface of HPIV3 HN as observed in its DANA-complexed form (10) showing the relative locations of residues H552 and E549 as well as residues R192, R424, R509, and Y530, which are involved in coordinating the ligand. H552, the dimer interface residue of interest, is labeled on both monomers. All strands within a given β-sheet and its symmetry-related counterpart in the opposing monomer are colored identically (sheet 1, red; sheet 2, orange; sheet 3, yellow; sheet 4, green; sheet 5, blue; sheet 6, mauve). Monomer A is shown as having a gray coil linking the secondary structure elements, while monomer B has a dark red coil linking the secondary structure elements. The DANA moiety and the selected residues within each monomer are shown in stick representation (carbon atoms, green; oxygen atoms, red; nitrogen atoms, blue). Large shaded spheres (gray within monomer A and dark red within monomer B) indicate the locations of the polypeptide segments in HPIV3 HN that are topologically equivalent to those that form the second receptor-binding site within NDV HN (25). The figure was generated from Protein Data Bank (1) entry 1V3D with MOLSCRIPT (8) and RASTER3D (14).

arate sites on HN may be responsible for the two activities. In the case of NDV, the ability of DANA and FANA to inhibit neuraminidase activity has long been known (13, 18, 19), and such an inhibition was proposed to explain the observed interference of these compounds with NDV plaque formation. Although FANA inhibited the hemagglutination of erythrocytes by NDV (13), it did not interfere with NDV binding to chicken embryo fibroblasts (19), implying that the functions of neuraminidase and binding may reside at separate sites. Initial crystallographic studies suggested that a single site on NDV HN was responsible for both activities (4); however, a more recent study has identified a second potential NDV HN receptor-binding site (25).

Our observations here regarding the effect of 4-GU-DANA and DANA on receptor binding of both HPIV3 HN and NDV HN provide insights into these issues. We have shown that both HPIV3 HN and NDV HN exhibit sensitivity to DANA in a receptor-binding assay (Fig. 4). However, this does not necessarily imply that receptor binding occurs at the site of DANA binding observed for HPIV3 HN and NDV HN (4, 10, 25). We note (Fig. 4) that in the case of NDV HN, residual receptor binding remained even at high concentrations of DANA, but this was not the case for HPIV3 HN. Furthermore, receptor binding in both wt NDV HN, which has an isoleucine at 175 that is equivalent to T193 in HPIV3 (Fig. 2), and an I175T variant NDV HN (data not shown) was only minimally sensitive to 4-GU-DANA. Taken together, these data are compatible with the presence of separate active sites in NDV HN, with one exhibiting both enzyme- and receptor-binding activities and the second exhibiting only receptor binding. The first site is sensitive to both DANA and 4-GU-DANA, while the second site is not affected by 4-GU-DANA and perhaps is affected only to a limited extent by the smaller molecule DANA.

A potential candidate for this second receptor-binding site is the position recently observed in the structure of NDV HN complexed with thiosialoside (25). Our observations further imply either (i) that such a second site does not occur in the case of HPIV3 HN, or if it does occur, (ii) that it has an affinity for DANA. The putative second receptor-binding site in NDV HN is formed within the groove of the dimer interface (25). This interface is somewhat different in HPIV3 HN in that the relative dispositions of the constituent monomers are different in these two structures (defined by an 18.4° rotation and 5.6-Å center-of-mass translation of one monomer with respect to its counterpart in the other dimer [10]). Should such a second receptor-binding site thus occur in the same vicinity in HPIV3 HN as in NDV HN, its atomic detail would have to be different from that of NDV HN.

We have previously shown that, for HPIV3, sialic acid analogs such as 4-GU-DANA may counteract infection by inhibiting receptor binding and viral entry rather than by inhibiting neuraminidase (22). It is thus possible that for HPIV3, such sialic acid analogs are feasible as antivirals; however, they would work not by interfering with virus release but by functioning as binding and entry inhibitors. For NDV, our results indicate that the opposite is likely to be the case; these compounds inhibit neuraminidase and reduce infection in culture but do not completely inhibit binding or block viral entry. We note, however, that the concentration of 4-GU-DANA that is required to inhibit NDV neuraminidase is far higher than that for influenza virus (24), for which the inhibition of neuraminidase during the release process accounts for the clinical effectiveness of 4-GU-DANA as an antiviral agent. It is possible that sialic acid analogs that are specifically designed to inhibit the NDV neuraminidase's active site, and thereby are more effective inhibitors of the enzyme, may inhibit virion release as they do in the case of influenza virus.

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