A release-competent influenza A virus mutant lacking the coding capacity for the neuraminidase active site

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Both influenza A virus surface glycoproteins, the haemagglutinin (HA) and neuraminidase (NA), interact with neuraminic acid-containing receptors. The influenza virus A/Charlottesville/31/95 (H1N1) has shown a substantially reduced sensitivity to NA inhibitor compared with the A/WSN/33 (H1N1) isolate by plaque-reduction assays in Madin–Darby canine kidney (MDCK) cells. However, there was no difference in drug sensitivity in an NA inhibition assay. The replacement of the HA gene of A/WSN/33 with the HA gene of A/Charlottesville/31/95 led to a drastic reduction in sensitivity of A/WSN/33 to NA inhibitor in MDCK cells. Passage of A/Charlottesville/31/95 in cell culture in the presence of an NA inhibitor resulted in the emergence of mutant viruses (delNA) whose genomes lacked the coding capacity for the NA active site. The delNA mutants were plaque-to-plaque purified and further characterized. The delNA-31 mutant produced appreciable yields (~10^6 p.f.u./ml) in MDCK cell culture supernatants in the absence of viral or bacterial NA activity. Sequence analysis of the delNA mutant genome revealed no compensatory substitutions in the HA or other genes compared with the wild-type. Our data indicate that sialylation of the oligosaccharide chains in the vicinity of the HA receptor-binding site of A/Charlottesville/31/95 virus reduces the HA binding efficiency and thus serves as a compensatory mechanism for the loss of NA activity. Hyperglycosylation of HA is common in influenza A viruses circulating in humans and has the potential to reduce virus sensitivity to NA inhibitors.

Introduction

Both influenza A virus surface glycoproteins, the haemagglutinin (HA) and neuraminidase (NA), interact with neuraminic acid-containing receptors. The HA binds to neuraminic acid-containing receptors and the NA destroys HA receptors by cleaving the terminal neuraminic acid residues from adjacent oligosaccharide chains. Both viral glycoproteins are sialylated by cellular enzymes, and the activity of viral NA is required to prevent self-aggregation of progeny virions and to promote release from the cellular membrane (Colman, 1994). Each subunit of the NA homotetramer consists of a cytoplasmic tail, a transmembrane domain, a stalk and a head region. The crystal structure of the NA head, which contains the enzyme active site, has been extensively studied and used in rational drug design (Colman, 1994). The recently developed NA inhibitors zanamivir and oseltamivir have demonstrated therapeutic benefit in clinical trails, and the inhibitor RWJ-270201 (BCX-1812) is undergoing clinical evaluation (Gubareva et al., 2000).

Influenza viruses have demonstrated a broad range of sensitivities to NA inhibitors in cell culture, despite the efficient...
inhibition of the NA activities (Woods et al., 1993; Zambon & Hayden, 2001). Prolonged passage in MDCK cells in the presence of NA inhibitor can lead to the emergence of drug-resistant viruses that have acquired amino acid substitutions in the NA active site (McKimm-Breschkin, 2000). Additionally, amino acid changes in the HA are frequently detected in NA inhibitor-resistant mutants selected in vitro and often precede drug-selected changes in the NA (McKimm-Breschkin, 2000). Influenza viruses with substitutions in the NA active site and reduced sensitivity in enzyme inhibition assays have occasionally been recovered from people treated with NA inhibitors (Gubareva et al., 1998, 2001a; Treanor et al., 2000; Whitley et al., 2001). In accordance with the observed interdependence of the HA and NA functions during influenza virus propagation (Yang et al., 1997; Kaverin et al., 1998; Mitnau et al., 2000; Wagner et al., 2000; Baigent & McCauley, 2001), the decreased efficiency of HA binding to the cellular receptors is believed to facilitate the release and spread of the virus when its NA activity is low. To this end, previous studies by others have shown that NA-lacking mutants, generated by supplying the bacterial NA and antibodies to viral NA, undergo multiple rounds of replication only in the presence of exogenous NA or after sonication of membrane-attached virus aggregates (Liu & Air, 1993; Liu et al., 1995). It has also been shown that substitutions in the HA of the NA-lacking mutants can provide compensation for the loss of the endogenous NA activity (Hughes et al., 2000).

Previously, we have reported that clinical isolates of influenza A (H1N1) viruses A/Charlottesville/31/95 and A/Charlottesville/28/95 passed in MDCK cells in the presence of NA inhibitors BCX-1812 or oseltamivir carboxylate acquired no point mutations in either the HA or NA genes. Yet, with the use of RT–PCR analysis, we were able to detect the emergence and accumulation of the defective RNA segments encoding the NA but not HA in these viruses (Nedyalkova et al., 2002). We suggested that the accumulation of defective NA genes was a consequence of the reduced sensitivity of the virus to NA inhibitors by substituting the HA gene with the sensitivity to NA inhibitors by substituting the HA gene (McKimm-Breschkin, 2000). Additionally, resistant viruses that have acquired amino acid substitutions in the presence of NA inhibitor can lead to the emergence of drug-selected changes in the NA (McKimm-Breschkin, 2000). The reasortant virus was produced by transfection of cells with seven plasmids containing the genome segments of A/WSN/33 and one plasmid containing the HA gene of A/Charlottesville/31/95. The plasmid pHW-HA31 containing the cDNA of the HA gene of A/Charlottesville/31/95 was constructed by RT–PCR amplification of the viral RNA. cDNA was amplified by PCR using oligonucleotide primers containing the site of digestion for BsmBI. To amplify the HA gene, the primers RGHA1 (5′ TATTCGGTCCTCAGG- GACCAGAGGCAAAAGAT 3′) and RGN2/H2A (5′ ATATCGTCTCGTATTAGTAGAAAACGGTGTTT 3′) were used. Viral sequences are underlined and restriction sites are shown in bold.

**Method**

**Compounds.** The influenza virus NA inhibitor BCX-1812 (RWJ-270201) was provided by BioCryst.

**Viruses and cells.** A/WSN/33 (H1N1) was generated from eight plasmids containing the entire virus genome (see below). Influenza A virus isolates A/Charlottesville/31/95 (H1N1) and A/Charlottesville/28/95 (H1N1) were from the repository at the University of Virginia. Madin–Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM) supplemented with 10% foetal bovine serum. Viruses were grown in monolayers of MDCK cells in MEM supplemented with 0.3% BSA and 1 μg/mL TPCK-treated trypsin. A/Charlottesville/31/95 underwent 18 passages in the presence of BCX-1812. The clinical isolate A/Charlottesville/28/95 (H1N1) underwent five passages in MDCK cells in the presence of oseltamivir carboxylate (Nedyalkova et al., 2002).

All comparisons of properties were made between the properties of the mutant and its wild-type (wt) virus grown in MDCK cells for the same number of passages in the absence of the NA inhibitor.

**Gene reassortment using reverse genetics.** The eight plasmids containing the cDNA of the virus A/WSN/33 (H1N1) were kindly provided by Dr Robert G. Webster at St Jude Children’s Research Hospital (Hoffmann et al., 2000). Transfection of co-cultivated 293T and MDCK cells was performed as described (Hoffmann et al., 2000). The reassortant virus was produced by transfection of cells with seven plasmids containing the genome segments of A/WSN/33 and one plasmid containing the HA gene of A/Charlottesville/31/95. The plasmid pHW-HA31 containing the cDNA of the HA gene of A/Charlottesville/31/95 was constructed by RT–PCR amplification of the viral RNA. cDNA was amplified by PCR using oligonucleotide primers containing the site of digestion for BsmBI. To amplify the HA gene, the primers RGHA1 (5′ TATTCGGTCCTCAGG-GACCAGAGGCAAAAGAT 3′) and RGN2/H2A (5′ ATATCGTCTCGTATTAGTAGAAAACGGTGTTT 3′) were used. Viral sequences are underlined and restriction sites are shown in bold.

**NA activity measurement.** A standard fluorimetric assay with minor modifications (Potier et al., 1979) was used to measure NA activity of the virus in cellular supernatants and in concentrated virus preparations. NA activity was measured with 2-(4-methylumbelliferyl) α-D-N-acetyl neuraminic acid (Sigma) as a substrate at a final concentration of 100 μM. The reactions were carried out in 33 mM MOPS buffer, pH 6.5, supplemented with 4 mM CaCl2. The assessment of the virus sensitivity to NA inhibitors was performed in an NA inhibition assay, as described previously (Gubareva et al., 2001a). The IC50 value reflects the concentration of drug that reduces the NA activity by 50%.

**Plaque-reduction assay.** Virus sensitivity to NA inhibitors was tested in a standard plaque-reduction assay, as described previously (Gubareva et al., 2001a). Briefly, monolayers of MDCK cells were infected with virus at an m.o.i. of approximately 30 p.f.u. per well. After 1 h adsorption, the virus inoculum was discarded and cells were overlaid with an MEM–agarose mixture containing trypsin and the NA inhibitor. The diameter of plaques and their number were determined after 72 h of incubation at 36 °C. Drug sensitivity was assessed by two criteria: the concentration of inhibitor causing 50% reduction of the plaque diameter (IC50 plaque diameter) and the concentration causing reduction of the plaque number by 50% (IC50 plaque number) compared with the control without inhibitor.

**Immunofluorescent staining.** Efficiency of virus adsorption was examined as described previously for NA inhibitor-resistant viruses (Blick et al., 1998) with minor modifications. MDCK cells were seeded on tissue
culture glass slides (Becton Dickinson). Confluent monolayers were washed with cold PBS and inoculated with virus. Adsorption was allowed to proceed for 20, 40 and 60 min on ice, followed by removal of the inoculum and washing the cells. As a positive control for maximum adsorption, the virus inoculum was removed after incubation for 60 min on ice followed by additional incubation at 37 °C for 60 min. The inoculum was removed and infected cells were incubated in a CO₂ incubator at 37 °C for 7 h followed by washing with PBS. The washed cells were air-dried and fixed with cold acetone for 10 min. The cells were stained with a mixture of anti-NP mAbs (kindly provided by Dr Robert G. Webster, St Jude Children’s Research Hospital, Memphis, TN, USA), followed by the addition of FITC-labelled goat anti-mouse immunoglobulins. The stained cells were viewed with a fluorescence microscope (Olympus BH-2 RFCA).

**Viral protein analysis.** Radioisotopic labelling of viral proteins was performed during virus propagation in MDCK cells. Four hours after infection, cells were starved in methionine-free medium for 30 min. The infected cultures were then incubated in maintenance medium containing half the usual concentration of unlabelled methionine and [³⁵S]methionine (25 μCi/ml) for 24 h. The growth medium did not contain trypsin to prevent proteolytic cleavage of the HA precursor (HA₀) into the two subunits, HA₁, and HA₂. The culture fluids were collected and clarified and the labelled virus was pelleted through a 25% sucrose cushion. Viral proteins in the pellets were analysed by SDS–PAGE under reducing and non-reducing conditions.

When specified, an aliquot of the concentrated virus was pretreated with bacterial sialidase (5 units/ml, from *Clostridium perfringens*; Glyko) for 2 h at 37 °C or with peptide-N-glycosidase F (N-glycanase, 0.5 units/ml; Glyko) overnight.

**Electron microscopy.** For electron microscopy studies, confluent monolayers of infected cells were washed three times with PBS, fixed with 2% glutaradehyde in PBS for 2.5 h at 4 °C and post-fixed with 2% osmium tetroxide. The fixed cells were dehydrated with increasing concentrations of ethanol (50–100%) and embedded in a mixture of epoxy resin. Ultrathin sections of cells were cut with a diamond knife on a Sorvall MT 6000 Ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined using a Philips EM301 electron microscope at 80 kV.

**RT–PCR and sequence analysis.** Extraction of viral RNA was performed as described previously (Gubareva et al., 2001a). The sequences of the primers used for PCR amplification of the eight segments of the influenza A (H1N1) viruses and for their sequence analysis are available on request.

The synthetic primer 5’ AGCAAAAGCAGG 3’ was used to generate cDNA with reverse transcriptase (Promega). cDNA was amplified by a standard PCR method and purified with the QIAquick PCR purification kit (Qiagen). Purified PCR products were sequenced using Taq Dye Terminator chemistry, according to the manufacturer’s instructions (Applied Biosystems) and then analysed on an ABI 373 DNA sequencer (Applied Biosystems) at the Center of Biotechnology at the University of Virginia. Sequencher 4.0 software (Gene Codes Corporation) was used for the analysis of nucleotide sequence data. The analysis did not include the sequence of the 30 nucleotides at the 3’ and 5’ end of each segment.

**Results**

**Sensitivity to NA inhibitors**

To investigate the mechanisms that allow compensation for the loss of the NA activity, we initially compared the drug sensitivities of the clinical isolate A/Charlottesville/31/95 (H1N1) and the laboratory-adapted virus A/WSN/33 (H1N1). In the plaque-reduction assays, the IC₅₀ plaque number for the A/WSN/33 virus was 0.1 μg/ml of BCX-1812. However, no inhibition was detected for the A/Charlottesville/31/95 virus at a 100-fold higher concentration (IC₅₀ plaque number > 10 μg/ml). Additionally, the diameter of plaques produced by A/WSN/33 was reduced by 50% at a drug concentration at least 100-fold lower than that required for the A/Charlottesville/31/95 virus (IC₅₀ plaque size < 0.0001 μg/ml and 0.01 μg/ml, respectively). When tested in the NA enzyme inhibition assay, the viruses A/WSN/33 and A/Charlottes-
Fig. 2. For legend see opposite.
Drug sensitivity of the reassortant virus

To assess the HA impact on the virus sensitivity to NA inhibitor, we performed a gene reassortment with the use of a reverse genetics technique (Hoffmann et al., 2000). The reassortant virus carrying the HA gene of A/Charlottesville/31/95, with the remaining genes from A/WSN/33, showed reduced sensitivity to BCX-1812 (IC$_{50}$ plaque number and diameter > 10 µg/ml). These results indicated that the HA of A/WSN/33 was largely responsible for the high drug sensitivity of this virus in cell culture.

Isolation of NA-lacking mutants

In our previous study, we observed that prolonged passage of A/Charlottesville/31/95 in the presence of high concentrations of BCX-1812 resulted in the rapid accumulation of defective NA genes (Nedyalkova et al., 2002). The uncloned virus preparation contained two kinds of the NA genes, full-sized and defective ones (Nedyalkova et al., 2002). If our assumption was correct that the HA of A/Charlottesville/31/95 plays a key role in lowering the virus requirements for the NA activity, we should be able to recover a mutant containing no full-sized NA genes, with an associated loss of NA activity. In the current studies, we therefore sought to isolate an NA-lacking mutant and to study its properties. Three of 19 randomly picked plaques were produced by viruses that contained no full-sized NA genes. The progeny of one of these clones was plaque-to-plaque purified and designated as the delNA-31 mutant. The other NA-lacking mutant (delNA-28) was generated by a similar approach when a clinical isolate of A/Charlottesville/28/95 (H1N1) was passaged in the presence of oseltamivir carboxylate (Nedyalkova et al., 2002). This NA-lacking mutant was used to confirm the observations made for the delNA-31 mutant.

Characterization of the NA-lacking mutants

The defective NA gene of the delNA-31 mutant encoded a 95 amino acid polypeptide of residues comprising the cytoplasmic tail, the transmembrane domain and the stalk of the NA; however, this defective gene did not contain genomic information encoding the head carrying the NA active site. Similarly, the defective NA gene of the delNA-28 mutant encoded a peptide of 74 amino acids. Importantly, sequence analysis detected no changes in the HA of the delNA-31 and the delNA-28 mutants compared with their wild-type counterparts. In order to identify any possible extragenic mutations in the genome of the delNA-31 mutant, we analysed the sequences of the remaining six genomic segments of this mutant and compared them with the wild-type. Only two amino acid substitutions (Ser-409 → Asn and Leu-531 → Ile) were detected; they were located in segment 3, which encodes the polymerase subunit PA. Sequence analysis of segment 3 of the delNA-28 mutant indicated no mutations.

As expected, the loss of coding capacity for the NA active site led to the loss of NA activity. When tested in the highly sensitive fluorimetric assay, NA activity was not detected in either the cell supernatants containing $10^6$ TCID$_{50}$/ml of the delNA-31 mutant or in the approximately 100-fold-concentrated virus preparation. The NA activity of the wild-type virus preparation ($10^6$ TCID$_{50}$/ml) was easily detected under the same experimental conditions. The delNA-31 mutant produced small plaques (approximately 1 mm) in MDCK cell monolayers and the addition of NA inhibitor into the agarose overlay (at 100 µg/ml) affected neither the size nor the number of plaques (not shown).

In vitro replication of the NA-lacking mutant

It has been demonstrated in previous studies (Liu et al., 1995) that the NA-lacking NWS-Mvi mutant produces large aggregates at the cell surface and in cytoplasmic vacuoles. A massive virus aggregation was also demonstrated when A/WSN/33 was propagated in the presence of NA inhibitor FANA (Palese & Combs, 1976) and BCX-1812 (Fig. 1B). In contrast, the aggregation of the progeny of the delNA-31 mutant was inferior and restricted to production of small virus clumps at the surface of the infected MDCK cells (Fig. 2C, D).

To evaluate the growth characteristics of the delNA-31 mutant, MDCK cells were inoculated with either wild-type or the mutant virus at equal m.o.i. values. The titres of the delNA-31 in the cell supernatants were approximately 100-fold lower than those of the wild-type at 60 and 72 h after infection (Fig. 3a). Of note, the titres of the delNA-31 virus associated with the cell membranes were approximately 10-fold lower than those of the wild-type virus (Fig. 3b). Therefore, the delNA-31

Fig. 2. Electron micrographs of MDCK cells in the absence of NA inhibitor. (A) Uninfected cells; (B) budding and release of A/Charlottesville/31/95; (C, D) limited aggregation of virions at the surface of cells infected with the delNA-31 mutant virus. Bar, 1 µm.
Fig. 3. Growth curves of the wild-type virus (■) and the delNA-31 mutant (○). MDCK cells were infected with either the wild-type A/Charlottesville/31/95 or the mutant virus, and virus yields were harvested at the indicated times. The virus titres in the supernatant (a) and the cell-associated (b) fractions were determined. M.o.i., 0.01 TCID_{50}/cell.

Fig. 4. Adsorption of A/Charlottesville/31/95 (a, b) and the delNA-31 mutant (c, d) to the MDCK cell monolayer. In (a) and (c), adsorption was carried out at 0 °C for 20 min. In (b) and (d), adsorption was carried out at 0 °C for 60 min, followed by adsorption at 37 °C for 60 min. Adsorption was followed by removal of the inoculum and incubation at 37 °C for 7 h. The samples were then processed for immunofluorescence, and infected cells were detected using a mixture of anti-NP monoclonal antibodies.
Drug-selected NA deletion mutant

(a) wt 31 delNA-31 wt 28 delNA-28

HA0
NA
NP
M1

(b) wt 31 delNA-31 wt 28 delNA-28

HA0
NA dimer
NP
M1

(c) wt 31 delNA-31

HA0
NA
NP
NA*

+PNG-F +PNG-F

(d) wt 31 delNA-31

HA0
NP
M1

+Sialidase

HA0-sia

Fig. 5. Protein profile analysis by SDS–PAGE. Plates of MDCK cells were infected with the wild-type viruses and the corresponding delNA mutants in the absence of trypsin. Four hours after infection, 25 µCi [35S]methionine was added to the cells and the supernatants were harvested at 20 h post-infection. After the cellular debris had been removed, the virus particles were pelleted and subjected to electrophoresis on SDS–polyacrylamide gels. (a) 12% SDS–PAGE under reducing conditions. Lane 1, A/Charlottesville/31/95; lane 2, delNA-31 mutant; lane 3, A/Charlottesville/28/95; lane 4, delNA-28 mutant. All viruses contained HA0, NP and M1 proteins, but NA could be detected only in the wild-type viruses. NP migrated as two bands. (b) 12% SDS–PAGE under non-reducing conditions. Lane designations are the same as those in (a). The mobility of HA0 from the delNA viruses was reduced in comparison to those of the wild-type viruses. The NA monomers and NA dimers were seen in the preparations of the wild-type viruses but not in those of the delNA mutants. (c) 12% SDS–PAGE under reducing conditions following pretreatment. Lanes 1, A/Charlottesville/31/95; lane 2, A/Charlottesville/31/95 pretreated with PNG-F; lane 3, delNA-31 mutant; lane 4, delNA-31 mutant treated with PNG-F. A shift in the mobilities of the HA0 monomers of both viruses was observed following pretreatment (HA0*); the pretreated HA0 of both viruses exhibited similar mobilities. A shift in the mobility of the NA monomer was also seen in the wild-type virus (NA*) following pretreatment. (d) 10% SDS–PAGE under non-reducing conditions. Pretreatment of the wild-type virus and the delNA mutant with bacterial sialidase reduced the HA0 mobility of the delNA-31 mutant (HA0-sia → HA0) but not that of the wild-type virus, indicating the presence of terminal neuraminic (sialic) acid residues on the HA0 of the NA-lacking mutant.

mutant was capable of release, although less efficiently compared with the wild-type.

Binding efficiency of the NA-lacking mutant

To confirm the reduced HA binding efficiency for MDCK cell receptors, we tested the viruses in an assay previously utilized by others to characterize NA inhibitor-resistant mutants (Blick et al., 1998). MDCK cells were inoculated with either the wild-type or the delNA-31 mutant at the same m.o.i. After time-controlled adsorption, the infected cells were incubated for 7 h and stained with antibodies against the viral nucleoprotein (NP). For the wild-type virus, 20 min of adsorption was sufficient to produce approximately 50% NP-positive cells compared with its control (Fig. 4a, b). In contrast, only 15% of cells were positive for NP in the delNA-31-infected monolayers after 20 min of adsorption (Fig. 4c) compared with its control (Fig. 4d). Therefore, these data indicate that the delNA-31 mutant demonstrated substantially reduced adsorption efficiency compared with the wild-type.

Sialylation of the HA

Next we analysed the wild-type viruses and the delNA mutants by SDS–PAGE. As expected, there were no bands corresponding to the full-sized NA in the lanes containing the delNA-31 and delNA-28 mutants under reducing (Fig. 5a) and non-reducing conditions (Fig. 5b). The electrophoretic mobilities of the HA0 (HA precursor) bands of the wild-type viruses were slightly greater than those of the delNA mutants (Fig. 5a); this difference in mobility was especially apparent under non-reducing conditions (Fig. 5b). To remove oligosaccharide
chains attached to asparagines of the viral glycoproteins, we pretreated virus preparations with peptide-N-glycosidase F (PNG-F). Removal of oligosaccharide chains was accompanied by a shift in the band corresponding to the HA₀ and NA of the wild-type virus. After treatment, the mobilities of the HA₀ of the wild-type and the delNA-31 viruses became equal (Fig. 5c). The pretreatment of viruses with exogenous sialidase (from *Clostridium perfringens*) also abolished the difference in the electrophoretic mobilities of the HAs (Fig. 5d). Therefore, the difference in the HA₀ mobilities of the wild-type and the delNA viruses appeared to be caused by the presence of terminal neuraminic acid moieties on the HA of the delNA mutants in the absence of NA activity.

**Discussion**

Here, we present direct evidence that the HA of a recent human influenza A (H1N1) virus plays a key role in its low sensitivity to NA inhibitors in MDCK cells. Moreover, the reduced need for NA activity led to the emergence of NA-lacking mutants when this virus was passaged in cell culture in the presence of an NA inhibitor. Baigent *et al.* (1999) showed that sensitivity to NA inhibitors of naturally occurring avian influenza A viruses is determined by both the HA and NA. To demonstrate the role of HA in the human virus sensitivity to NA inhibitor, we used a reverse genetics technique to generate a reassortant virus, carrying the HA gene from A/Charlottesville/31/95 (H1N1) and the remaining genes of A/WSN/33 (H1N1). This approach ensured that no unwanted changes had been introduced into the genome of the reassortant virus that could affect the drug phenotype. We demonstrated that replacement of the HA gene of the highly drug-sensitive A/WSN/33 (H1N1) drastically reduced the sensitivity of A/WSN/33 in cell culture. Furthermore, we found that glycosylation of HA from the recent clinical isolate differed substantially from that of A/WSN/33. Analysis of the HA sequences of the A/WSN/33 and A/Charlottesville/31/95 isolates revealed that the recent human strain had twice as many potential glycosylation sites in its HA₁ subunit (eight compared with four). Some of these oligosaccharide chains reside in the vicinity of the RBS (i.e. Asn-129 and Asn-163; N1 numbering). HA hyperglycosylation is not confined only to the two clinical isolates used in the present study but is a characteristic trait of the recent human influenza viruses of H1N1 subtype (Inkster *et al.*, 1993). The regulatory function of the N-glycans on the HA molecule in virus growth and in its requirement for NA activity has been extensively investigated in several laboratories (Baigent *et al.*, 1999; Matrosovich *et al.*, 1999; Wagner *et al.*, 2000; Baigent & McCauley, 2001). Ohuchi *et al.* (1997) demonstrated that adsorption of erythrocytes to the viral HA could be reduced by the presence of oligosaccharide chains in the vicinity of the RBS, and the presence of uncleaved neuraminic acid moieties on these chains reduces the HA binding still further (Ohuchi *et al.*, 1997).

The question arises as to whether sialylation of the HA alone can render the virus resistant to NA inhibitors. The number of plaques produced by A/Charlottesville/31/95 in MDCK cells was not affected by the presence of the NA inhibitor at the highest concentration tested (10 μg/ml). This result demonstrated that this virus had over 100-fold reduced sensitivity to NA inhibitor compared with A/WSN/33 in cell culture. Nevertheless, the diameter of plaques produced by A/Charlottesville/31/95 was inhibited at lower drug concentrations (0.01 μg/ml) indicating a restricted spread of the virus under agarose overlay. None the less, in liquid media, the NA independence of A/Charlottesville/31/95 allowed the emergence of a mutant completely lacking the coding capacity for the NA active site. Therefore, it was important to investigate whether the NA-lacking mutant had acquired any additional compensatory mechanisms besides the sialylated oligosaccharide chains in the vicinity of the RBS. We found that the genome of the mutant contained only the defective NA genes that arose as a result of a massive internal deletion. In this respect, it was similar to the NA-lacking mutants generated by others under conditions when the endogenous NA activity was not required (Liu & Air, 1993; Hughes *et al.*, 2000, 2001). We did not attempt to detect the products of the defective NA genes in the infected cells or the delNA virus because the previous extensive studies by Yang *et al.* (1997) produced no evidence for the expression of defective NA genes in cells infected with the other NA-lacking mutant, NWS-Mvi (Yang *et al.*, 1997). Importantly, sequence analysis of the delNA-31 genome detected no amino acid substitutions, which could have provided additional compensation for the loss of NA activity. The only two amino acid substitutions found were in the PA polymerase subunit. No substitutions were detected in the PA of the other NA-lacking mutant (delNA-28) generated under similar conditions. Therefore, we believe that the lower binding efficiency of the delNA-31 mutant compared with the wild-type (Fig. 4) was directly by sialylation of the oligosaccharide chains of the HA (Fig. 5). The delNA-31 mutant was able to elute from infected cells and produced only small clumps of progeny virions at the cell surface (Fig. 2). Importantly, the delNA-mutant produced a substantial yield of infectious virus in the supernatant fraction, although not as high as the wild-type virus (Fig. 3a). The considerable NA independence of the delNA-31 mutant separates this virus from the other NA-lacking viruses generated on the basis of the NWS-G70c reassortant (H1N9) (Liu *et al.*, 1995; Hughes *et al.*, 2000). The difference in the number (four versus eight) of the oligosaccharide chains in the HA₉ of NWS-Mvi and delNA-31 could be one explanation. It is noteworthy that the egg-grown A/Texas/36/91 (H1N1) variant was highly sensitive to NA inhibitors in cell culture despite the presence of oligosaccharide chains in the vicinity of its RBS. A plausible explanation for this is the acquisition of amino acid substitutions in the RBS caused by egg-adaptation (Gubareva *et al.*, 2001a).
The emergence of NA-lacking mutants following virus passage in the presence of NA inhibitor provides additional evidence that NA activity is not obligatory when virus is propagated in vitro (Liu et al., 1995). However, the reduced need for NA activity demonstrated for the recent influenza A viruses in the present study does not necessarily imply that these viruses are resistant to NA inhibitors during replication in humans. The NA function in vitro is not restricted to the prevention of virus aggregation at the surface of infected cells, but is also needed to avoid virus entrapment by the respiratory tract secretions and thus elimination. Furthermore, the receptors present on MDCK cells do not adequately reflect those present on the human respiratory tract epithelium (Baum & Paulson, 1990; Govorkova et al., 1995). Treatment with NA inhibitors was beneficial for individuals experimentally infected with the egg-adapted influenza A/Texas/36/91 (H1N1) (Hayden et al., 1996, 1999), although the HA of this virus has the same number of potential glycosylation sites as A/Charlottesville/31/95. Thus, hyperglycosylation of the HA did not abolish the inhibitory effect of the NA inhibitors on the egg-adapted virus replicating in a human host. Whether non-egg-adapted viruses with the hyperglycosylated HA are less sensitive to NA inhibitors in humans remains to be seen.

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References


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