Evidence for Zanamivir Resistance in an Immunocompromised Child Infected with Influenza B Virus

Larisa V. Gubareva,1 Mikhail N. Matrosovich, Malcolm K. Brenner,1 Richard C. Bethell, and Robert G. Webster

Zanamivir, a neuraminidase inhibitor, has shown promise as a drug to control influenza. During prolonged treatment with zanamivir, a mutant virus was isolated from an immunocompromised child infected with influenza B virus. A hemagglutinin mutation (198 Thr→Ile) reduced the virus affinity for receptors found on susceptible human cells. A mutation in the neuraminidase active site (152 Arg→Lys) led to a 1000-fold reduction in the enzyme sensitivity to zanamivir. When tested in ferrets, the mutant virus had less virulence than the parent; however, it had a growth preference over the parent in zanamivir-treated animals. Despite these changes, the sensitivity of the mutant virus to zanamivir assessed by a standard test in MDCK cells was unaffected. These data indicate that the current methods for monitoring resistant mutants are potentially flawed because no tissue culture system adequately reflects the receptor specificity of human respiratory tract epithelium.

Methods

Patient. An immunocompromised 18-month-old girl developed influenza B infection following bone marrow transplantation for juvenile chronic myelocytic leukemia. She was treated with 6 mg of ribavirin every 12 h by continuous aerosolized delivery. When her clinical condition deteriorated, approval was obtained from the US Food and Drug Administration for individual use of a new influenza NA inhibitor, zanamivir [2], and treatment was started 6 days after the diagnosis of infection. Zanamivir was administered by nebulizer at a dosage of 16–32 mg in 1–2 mL of sterile water every 6 h, the highest dosage tested in preliminary clinical trials [3, 4]. Treatment with zanamivir was discontinued when her clinical status worsened. During the 2 weeks she was treated with zanamivir, she shed virus, which was detected by routine nasopharyngeal swabs for virus. The child died of respiratory failure 2 days after zanamivir treatment was discontinued.

Virus isolates. The diagnosis of influenza B infection was confirmed by immunofluorescence (direct fluorescent antibody assay) detected on endotracheal tube aspirates (ETAs) from the patient. The influenza virus was initially isolated from clinical material and propagated in MDCK cells for 3 passages prior to use in drug sensitivity and other tests.

Drug sensitivity assays. Sensitivity of virus isolates to zanamivir was determined by plaque reduction assay in the presence of the drug in an agar overlay [8] and in liquid cultures as described [5]. To measure NA sensitivity of virus isolates to the drug, an NA inhibition assay was performed [9]. To estimate the specific activity of the NA, concentrated purified viruses were adsorbed onto microplates. Half of the microplate wells were used to determine the amount of NA by ELISA utilizing polyclonal rabbit antiserum against NA and anti-rabbit horseradish peroxidase (HRP) conjugate, and half were used to determine NA activity. The amount of NA activity (fluorescent units/l U of optical adsorption produced

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1 Present affiliation: Department of Internal Medicine, University of Virginia, Charlottesville (L.V.G.); Center for Cell and Gene Therapy, Houston (M.K.B.).

Reprints or correspondence: Dr. R. G. Webster, Dept. of Virology/Molecular Biology, St. Jude Children’s Research Hospital, 332 N. Lauderdale, Memphis, TN 38101.

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Table 1. Isolation and characterization of influenza virus isolates from an immunocompromised patient.

<table>
<thead>
<tr>
<th>Day of zanamivir treatment</th>
<th>Sensitivity to zanamivir in MDCK cells IC50 (μM)a</th>
<th>Antigenic analysis (HAI titer)b</th>
<th>Grown in MDCK cells</th>
<th>Amino acid substitution in virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>160</td>
<td>80</td>
<td>198Thr</td>
</tr>
<tr>
<td>6</td>
<td>198Thr</td>
<td>152Arg</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>198Ile</td>
<td>152Arg</td>
<td>198Thr/le</td>
<td>198Thr/le</td>
</tr>
<tr>
<td>9</td>
<td>198Ile</td>
<td>152Arg</td>
<td>198Thr/le/le</td>
<td>152Arg/Arg</td>
</tr>
<tr>
<td>12</td>
<td>0.1</td>
<td>320</td>
<td>&lt;20</td>
<td>198Ile</td>
</tr>
<tr>
<td>15</td>
<td>198Ile</td>
<td>152Lys</td>
<td>198Thr/le</td>
<td>152Arg/152Arg/Lys</td>
</tr>
</tbody>
</table>

NOTE. Viruses from aspirates were isolated and propagated twice in MDCK cells before use in plaque-reduction assay and sequence analysis. Amino acid substitutions in bold indicate change at residue shown. NA, neuraminidase.

a Concentration of drug in agar overlay that causes 90% reduction in plaques produced by virus in absence of inhibitor.

b Hemagglutination (HA) and HA inhibition (HAI) assays were done according to WHO protocols.

c Aspirates were obtained from patient and used to propagate virus in MDCK cells and for sequence analysis; endotracheal tube aspirates before treatment were not available for sequence analysis; ND = not done.

Results

Sequence changes in HA and NA genes associated with zanamivir treatment. The prolonged shedding of virus in this case prompted us to analyze virus isolates for drug resistance. By use of a standard protocol (a plaque reduction assay in MDCK cells with zanamivir in the agar overlay), we found no indication of resistance for the day 12 isolate (table 1). This isolate also displayed a sensitive phenotype in liquid cultures of MDCK, Vero (African green monkey kidney), and primary rhesus monkey kidney cells (data not shown). When we compared the HA and NA sequences of the after-treatment isolates with those of the parent virus, we found substitutions in both surface glycoproteins. The HA mutation (198 Thr→Ile), first identified in the day 8 isolate, abolished a glycosylation site at Asn196, which is near the receptor binding site [14]. This mutation also altered the antigenic properties of the mutant virus as determined by the HAI assay with the reference antiserum B/Guangdong/893 (table 1). The NA mutation (152 Arg→Lys; N2 numbering), first identified in the day 12 isolate, occurred in the

by the substrate in ELISA) was estimated for the virus isolated before (parent) and after (mutant) zanamivir treatment.

Sequence analyses of virus isolates. Isolation of viral RNA from tissue culture supernatants and ETAs and reverse transcription–polymerase chain reaction (PCR) amplification were performed in accordance with the manufacturer’s instructions (RNeasy kit; Qiagen, Chatsworth, CA). Purified PCR products of hemagglutinin (HA) and NA genes were sequenced as instructed by the manufacturer (Taq Dye Terminator chemistry; Applied Biosystems, Foster City, CA) and then analyzed by DNA sequencer (model 373; Applied Biosystems). To estimate the relative proportions of the parent and mutant HA and NA genes in clinical material, PCR products were cloned into a plasmid vector followed by transformation of competent cells (TA cloning kit; Invitrogen, San Diego). Individual colonies carrying the gene of interest were randomly picked and subjected to sequence analysis.

Evaluation of receptor-binding properties. Virus affinity for soluble receptor analogues was evaluated in a competitive assay based on inhibition of binding between the solid phase–immobilized virus and a standard preparation of bovine HRP-labeled fetuin [10].

Virus binding to solid phase–attached MDCK cell membranes was assayed in a microplate adsorption assay as described elsewhere [11]. In brief, 0.04 mL of MDCK membrane preparation suspended in PBS to a final concentration of ~5 μg/mL total protein was incubated overnight at 4°C in 96-well polystyrene microplates and then washed with PBS. After the coating step, 0.1 mL of the blocking buffer (0.1% bovine serum albumin in PBS) was added per well and incubated for 1 h at 37°C to block nonspecific binding of viruses to the plastic.

The blocking buffer was discarded, and 0.04 mL of serial 2-fold dilutions of the purified virus in blocking buffer was incubated in plate wells for 2 h at 4°C. The plates were rinsed 3 or 4 times with ice-cold washing buffer (0.2× PBS–0.02% Tween 80) and incubated with 0.05 mL/well of fetuin–HRP conjugate in PBS–0.02% Tween 80 for 1 h at 4°C. Unbound conjugate was removed by washing with wash buffer, and the amount of bound conjugate reflecting the amount of the virus present in the wells was quantified using o-phenylenediamine as a substrate.

As a positive control of maximal virus binding, the nonspecific virus attachment to the plastic was measured. The wells without coating were used, the blocking step was omitted, and the viruses were added to the plate in PBS instead of blocking buffer. All other steps of the assay were the same as described above.

Receptor-binding specificity of the viruses was also determined by HA inhibition (HAI) assay using erythrocytes from different animal species [12].

Infection in ferrets. Female ferrets (0.4–0.7 kg) were anesthetized intramuscularly with 0.5 mL of ketamine hydrochloride (Ketalar; 100 mg/mL; Parke-Davis, Morris Plains, NJ) and infected by intranasal instillation of the virus in 1 mL of normal saline. Virus solution was prepared by mixing the parent and mutant viruses at a ratio of ~1:60 (107 and 104.8 pfu/mL, respectively). Ferrets were treated intranasally with 0.75 mg/kg/dose (50 μL) of zanamivir. Treatment was 4 h before virus challenge, 4 h after challenge, and twice daily for 5 days as described [13]. For sample collection, animals were anesthetized intramuscularly with ketamine hydrochloride. Nasal washings used sterile physiologic saline instilled in each nostril. Virus in nasal washings was propagated once in MDCK cells and used for sequence analysis of the NA gene.
enzyme’s active site [15], creating the potential for modified NA sensitivity to zanamivir (table 1).

To ensure that the mutations we identified were not the result of selection of virus variants by a new host cell system [16], we analyzed the HA and NA sequences of the virus from the original ETAs. Partial sequence analyses confirmed the presence of the HA mutation (198 Thr→Ile) in specimens from day 8 of treatment, although the parent virus was also detected (table 1). The NA mutation (152 Arg→Lys) was detected in virus from ETAs obtained after 2 weeks of zanamivir treatment but not in earlier specimens. The same mutation was not detected in virus from the ETA sample at day 12 of treatment, even though this mutation was predominant in the virus population after passage in MDCK cells. Thus, the presence of the HA and NA mutants was confirmed in posttreatment clinical specimens prior to their culture in vitro, but the possibility cannot be excluded that passage in MDCK cells resulted in more mutant virus in the virus population.

Because Arg152 in NA is conserved in all sequenced influenza A and B viruses, where it forms a hydrogen bond to the acetyl-amine of sialic acids bound at the active site [17], we hypothesized that substitution of this NA residue could reduce enzyme activity. Parallel measurement of NA activity in parent and mutant virus preparations showed that the mutant enzyme activity in vitro was 3%–5% of the enzyme activity detected for the parent. In addition, the NA of the 12-day isolate was ~1000-fold less sensitive to zanamivir than was the parent enzyme in an in vitro inhibition assay (figure 1). Thus, the mutant virus isolated after 12 days of treatment contained NA with reduced sensitivity to the drug in the inhibition assay; however, it was as sensitive as the parent to the drug in MDCK cell culture.

Alteration of the mutant’s receptor binding properties. To explain the difference between the results from inhibition assays and those in MDCK cells, we asked whether the HA mutation was responsible for the zanamivir-sensitive phenotype exhibited by the mutant virus in MDCK cells. We wanted to know if the HA mutation could reduce viral sensitivity to zanamivir in the respiratory tract of the patient but have an opposite effect in the MDCK cell system. Reduction of the HA gene affinity for cellular receptors is one of the mechanisms postulated to reduce influenza virus sensitivity to NA inhibitors [18, 19]. Previous studies have shown that influenza virus receptors on the surface of respiratory tract epithelium are glycoconjugates containing the terminal NeuAca2-6Gal moiety (Siaα2-6Gal) [20]. We asked whether the HA mutation affected the interaction of the virus with substrates bearing this type of receptor determinant by measuring the binding affinity of the virus for sialic acid and sialyloligosaccharides and its ability to agglutinate erythrocytes carrying receptors with different linkages between the terminal sialic acid and the penultimate sugar (table 2). The mutant had a ≥10-fold lower binding affinity for 6′-sialyl-(N-acetyllactosamine), an essential sialo-sugar determinant of the cell-surface receptors of human influenza A and B viruses [22], and was unable to agglutinate human erythrocytes modified to bear predominantly Siaα2-6Gal receptors, which were readily agglutinated by parent virus (table 2). The reduced affinity of the mutant HA gene for Siaα2-6Gal receptors could aid virus release from human respiratory tract epithelium and, therefore, reduce virus dependence on NA function. Unlike the parent virus, the mutant agglutinated equine erythrocytes bearing predominantly Siaα2-3Gal receptors, although the difference in affinity of both viruses for low-molecular-mass analogue, 3′-sialyllactose, was only marginal. This apparent inconsistency indicates that the oligosaccharide chain attached to Asn196 of the parent virus HA gene does not directly affect the binding of the sialic acid and penultimate galactose of low-molecular-mass sialosides but sterically interferes with the recognition by the virus of Siaα2-3Gal determinants fixed in a context of macromolecular receptor [22]. A loss of this site by the mutant abrogates interference and substantially enhances virus binding to Siaα2-3Gal-containing cells.

MDCK cells contain receptors with both α2-6 and α2-3 linkages [12]. We prepared membranes from MDCK cells and compared parent and mutant virus attachment to them in a solid-phase adsorption assay. The mutant virus had a higher affinity for MDCK cell membranes than did the parent (figure 2A). Together, these results demonstrate that substitution of HA residue 198 leads to a reduced viral affinity for human receptors and a concomitant increase in affinity for MDCK cell receptors. Therefore, increased affinity of the mutant virus for MDCK cell receptors and reduced NA activity could be responsible for the zanamivir-sensitive phenotype in this cell system.

Growth preference of the mutant virus in ferrets treated with zanamivir. To determine whether the mutations increase the rate of replication in vivo of the mutant virus relative to the parent virus in the presence of zanamivir, we took advantage...
Table 2. Characterization of receptor-binding properties of the virus before and after zanamivir treatment.

<table>
<thead>
<tr>
<th>Binding of monovalent receptor analogues, $K_{\text{aff}}$ (mM)</th>
<th>Parent</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N$-acetylneuraminic acid</td>
<td>0.94 ± 0.2</td>
<td>&gt;2</td>
</tr>
<tr>
<td>6-sialyl-(N-acetyllactosamine)</td>
<td>0.084 ± 0.02</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>3-sialyllactose</td>
<td>0.17 ± 0.02</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

Hemagglutination with erythrocytes (titer)

<table>
<thead>
<tr>
<th></th>
<th>Parent</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken$^b$ (Sia$<em>{a2-6}$Gal and Sia$</em>{a2-3}$Gal)</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Human (NDV-treated$^c$, Sia$_{a2-6}$Gal)</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>Equine (Sia$_{a2-3}$Gal)</td>
<td>0</td>
<td>64</td>
</tr>
</tbody>
</table>

$^a$ Binding affinity constants ($K_{\text{aff}}$, formally equivalent to dissociation constants of virus/receptor analogue complexes) were determined in competitive solid-phase assay. High $K_{\text{aff}}$ corresponds to low affinity [10].

$^b$ Composition of linkage between sialic acid residues and their adjacent oligosaccharides on surface of native chicken and equine erythrocytes [12].

$^c$ Human erythrocytes were modified to contain predominantly Sia$_{a2-6}$Gal receptors by treatment with Newcastle disease virus (NDV) to specifically destroy Sia$_{a2-3}$Gal receptors [21].

of the fact that humans and ferrets share influenza virus receptors of similar specificity, namely, Sia$_{a2-6}$Gal [23]. We assumed that virus with NA resistant to zanamivir should have growth preference over the parent virus in the presence of the inhibitor, whereas the parent virus should outgrow the mutant in untreated animals.

Preliminary experiments with mutant and parent virus demonstrated that larger quantities of mutant virus were required to infect ferrets and that even with a very high inoculum, the nasal wash virus titers were substantially lower in animals infected with the mutant virus. To compensate for the lower rate of replication of the mutant virus in the absence of zanamivir, we coinfected ferrets with the parent and mutant viruses at a ratio of ~1:60 pfu. Virus in ferret nasal washings was propagated in MDCK cells, and the NA sequence of the resulting virus population was analyzed. The virus isolated on day 6 from untreated animals contained predominantly the parental sequence (table 3). In contrast, samples collected from zanamivir-treated ferrets had mutant virus (Arg152). These results indicate that in vivo treatment with zanamivir increased the relative rate of replication of the mutant virus compared with the parent virus, although the passage of the virus in MDCK cells before sequencing prevented the magnitude of this growth advantage from being quantified.

**Discussion**

Effective antivirals are essential to treat immunosuppressed recipients of bone marrow transplants because such persons are acutely susceptible to community respiratory viruses, which are associated with substantial morbidity and mortality [24]. The influenza NA inhibitors could provide an option for treatment of influenza infection in the near future. In our study, the antiviral NA inhibitor zanamivir was not available for treatment until 7 days after influenza B infection was diagnosed in the patient, which may have reduced the treatment efficacy. In this
patient, we were unable to determine whether zanamivir treatment reduced the virus load because quantification was not done before the samples were frozen. However, the continuous viral shedding during 15 days of treatment indicates incomplete viral clearance. Long-term shedding of influenza viruses in immunocompromised patients [25] illustrates the need for careful monitoring of virus isolates for potential drug resistance.

Although the mutants described here are from a single patient, the molecular changes resembled those observed by zanamivir selection in vitro [5–7, 9, 18]. The in vitro studies identified the selection of mutants with reduced dependence on NA function as a result of their HA gene’s reduced affinity for cellular receptors [18, 19]. The same mechanism is likely involved in selection of virus variants in the patient described, given that an influenza B virus with a mutation at the HA site emerged early in treatment (8 days). This mutation caused a reduction in the affinity of HA for Sia2-6Gal receptors. Viruses with reduced affinity for cellular receptors could be released from infected cells without the need for significant NA activity and could thereby escape the antiviral effect of zanamivir.

During the second, later phase of selection, the emergence of variants containing an NA mutation (152 Arg→Lys) that reduced the enzyme sensitivity to zanamivir was seen after prolonged drug administration (12–15 days). Previously, in vitro selection of zanamivir-resistant influenza B mutants resulted in the emergence of virus with a Glu119 substitution in NA [6]. Unlike Glu119, which is a “framework” residue that interacts with the guanidino group of the inhibitor but does not interact with the substrate [2], Arg152 is a “functional” residue that directly interacts with the sialic acid moiety and participates in the substrate binding [15]. Therefore, and in accordance with in vitro studies by Gubareva et al. [9], mutations in NA that lead to drug resistance could occur at functional residues in vivo. The replacement of Arg152 caused a significant reduction of NA activity, a finding previously reported for a recombinant NA gene carrying the same mutation [26].

The assays we performed in a variety of tissue culture systems (MDCK, Vero, and primary RMK cells) continued to indicate drug sensitivity of the after-treatment virus isolates. Although these results are in direct contrast with the viral enzyme resistance, we can reconcile the apparent discrepancy as a preference of the mutants for Sia2-3Gal receptors, which are present on the surface of MDCK and Vero cells [27] but not on that of human respiratory tract epithelium cells [20]. The optimal cell culture for surveillance systems to detect these mutants should, therefore, possess predominantly Sia2-6Gal receptors. The lack of a cell culture system to monitor the emergence of zanamivir-resistant mutants (or resistant mutants to other NA or HA inhibitors) may present difficulties for analysis of the emergence of drug-resistant viruses in clinical trials.

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References


