Rapid Selection of a Transmissible Multidrug-Resistant Influenza A/H3N2 Virus in an Immunocompromised Host

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Background. The overall impact of influenza virus infection in immunocompromised patients is largely unknown. Antigenic drift and genetic variations during prolonged influenza infection have been demonstrated. In this report we describe a multidrug-resistant H3N2 influenza virus isolated from an immunocompromised patient after 5 days of therapy.

Methods. Multiple nasal wash samples were collected from an infected patient, and viral isolates were characterized. Sensitivity to antiviral agents was evaluated. Fitness and transmissibility were assessed in ferrets and tissue culture.

Results. An in-frame 4–amino acid deletion emerged in the neuraminidase (NA) gene of an H3N2 virus after 5 days of oseltamivir therapy. No other changes in the NA or hemagglutinin genes were noted. Drug sensitivity assays revealed resistance to oseltamivir (>10-fold increase in 50% inhibitory concentration [IC50]) and reduction in sensitivity to zanamivir (3–7-fold increase in IC50 or 50% effective concentration). No change in fitness or transmissibility was observed.

Conclusions. An in-frame NA gene deletion was rapidly selected for in an immunocompromised patient, resulting in decreased sensitivity of the isolate to available NA inhibitors without a change in fitness or transmissibility. This finding has implications for our understanding of the emergence of antiviral resistance and treatment of patients with influenza A infection, especially those who are immunocompromised.

Despite progress in vaccine and antiviral development, influenza virus infection remains a major public health problem, with >36,000 deaths annually in the United States [1]. In pandemic years, increased numbers of infections and deaths have been noted [2–4]. The current novel H1N1 pandemic highlights the unpredictability of how new strains of influenza A virus can emerge and rapidly spread across the world [5]. These pandemic and novel viruses continue to pose health risks to individuals and a significant economic and public health challenge to healthcare providers and governments worldwide.

Antiviral agents are now commonly used to mitigate the impact of influenza infection. Many governments stockpile these agents, and in recent years their use by health care providers to treat influenza has increased. Owing to recent widespread resistance of seasonal A/H3N2 viruses to the adamantanes since 2005 [6, 7], neuraminidase (NA) inhibitors (NAIs) have increasingly become the drugs of choice [8]. Oseltamivir and the inhaled agent zanamivir have been shown to be clinically effective when given early in the course of disease, decreasing both duration of viral shedding and the occurrence of complications [9]. Until recently, resistance to these agents has been limited [10, 11], but...
during the 2008–2009 influenza season, nearly 100% of the circulating A/H1N1 viruses in many countries were found to contain the H275Y mutation (N1 numbering), conferring resistance to oseltamivir [12–14]. The recent A/H3N2 and the new pandemic A/H1N1 viruses, although resistant to the adamantanes, have retained sensitivity to NAIs, but oseltamivir resistance has been observed in a small but increasing number of cases [8, 11, 15]. Resistance to zanamivir has not been widely reported, but this drug has not been in widespread use and has limited usefulness in severely ill patients, owing to the nature of its administration as an inhalational agent and possible adverse effects [16, 17].

Because of the emergence of drug-resistant influenza viruses, the Centers for Disease Control and Prevention and the Infectious Disease Society of America recently adopted new guidelines for the treatment of influenza, recommending the use of zanamivir or combination therapy with oseltamivir with an adamantane to treat seasonal influenza empirically [8, 18]. NAI use will probably continue to increase during the current pandemic [19–21] and future influenza seasons, making it extremely important to investigate how antiviral resistance emerges.

Severe disease due to influenza infection occurs most often in individuals with underlying illnesses, and the current H1N1 pandemic continues to follow this trend [22, 23]. Immunocompromised individuals are one such high-risk group [24–27]. The impact of influenza on this growing population is still largely unknown. Prolonged illness and viral shedding, simultaneous infection with 2 subtypes, and viral genetic variation and antigenic drift during a single prolonged illness have been demonstrated in immunocompromised individuals [28–31].

Recently, a novel 4–amino acid deletion in the NA gene at positions 245–248 in an A/H3N2 virus was identified, conferring resistance to oseltamivir [32]. This deletion was found in the presence of other known NA resistance mutations in a virus isolated from a severely immunocompromised 3-year-old who had had a prolonged infection of nearly 1 year and had received a total of 107 days of NAI therapy. This patient received 3 months of continuous oseltamivir before the isolation of this mutant virus [31, 32]. The authors hypothesized that the NA deletion may have been detrimental to viral fitness, but this possibility was not fully evaluated in their study.

We describe the rapid emergence of an identical deletion at positions 245–248 in an immunocompromised adult patient infected with an influenza A/H3N2 virus (A/Bethesda/NIH12–0/2008). This is only the second reported instance of this deletion, and it is (to our knowledge) the first time it has been reported in the absence of other NAI resistance mutations. In addition, the deletion appeared after only 5 days of oseltamivir therapy. We evaluated the effect of this deletion on viral replication and transmissibility and observed no reduction in replicative fitness or in contact transmissibility in ferrets. Finally, we observed that this mutation was retained after transmission between mammalian hosts.

METHODS

Clinical case. A 43-year-old man with mantle cell lymphoma 6 months after allogeneic stem cell transplantation was admitted for rectal pain and a fluctuant mass. The patient had a history of cytomegalovirus reactivation and agranulocytosis. He received a dose of rituximab 5 days before hospital admission.

At hospital admission, the patient complained of dry cough and a mild sore throat. The next day he underwent incision and drainage of a perirectal abscess and was treated empirically with intravenous piperacillin-tazobactam. An Enterococcus species grew in abscess cultures, herpes simplex virus grew in a swab specimen culture, and appropriate therapy was administered.

Three days after admission, the patient developed fever (temperature, 38.5°C), coryza, fatigue, cough, and nasal discharge. Physical examination revealed no evidence of retained or secondary infection at the incision site, and findings of imaging studies were unremarkable. Laboratory diagnostic evaluation was notable for severe neutropenia (absolute neutrophil count, 120 cells/μL). A nasal wash sample was obtained, and a rapid influenza A test had a positive result. On the same day, treatment with oseltamivir phosphate was started (75 mg twice daily, taken orally).

The patient was enrolled in a clinical study of influenza (National Institutes of Health protocol “Influenza in Normal and Immunocompromised Hosts”), and a skilled respiratory therapist obtained nasal wash samples every other day during the patient’s infection. The patient manifested fever for a total of 4 days and upper respiratory symptoms for 2 weeks. He took oseltamivir phosphate for a total of 14 days. Two days after initial diagnosis, another nasal wash sample was obtained, with a positive rapid test and culture. After that time, all rapid test results were negative, but the patient continued to have positive influenza cultures for 12 days (Figure 1).

Sequence analysis. Reverse-transcription polymerase chain reaction (RT-PCR) and sequencing were performed on the primary specimens and viral isolates, as described elsewhere [33]. The influenza virus hemagglutinin and NA sequences determined in this study have been deposited in GenBank (GenBank accession numbers GU294117 and GU294118). Analysis of the initial nasal wash specimen included sequencing of 50 PCR products that were cloned using the TOPO TA Cloning kit (Invitrogen).

NA activity and antiviral susceptibility. NA activity and antiviral susceptibility were measured using 50 μL of 100 μmol/L methylumbelliferyl-N-acetyl neuraminic acid (Sigma) substrate, as described elsewhere [34]. An amount of virus that resulted in a signal of ~1 × 10^6 relative fluorescence units was added to serial dilutions of oseltamivir and zanamivir (Glaxo-
Figure 1. Viral shedding, diagnostics, and partial neuraminidase (NA) amino acid sequence. Nasal wash samples were collected from the patient every other day for 16 days. Nasal wash and shell vial cultures were performed for each sample (plus signs indicate positive results; minus signs, negative results). Nucleic acid sequencing of the NA gene was performed on all isolated viruses. The rapid test performed was the BinaxNow Influenza A & B test (Inverness Medical). The amino acid sequence is shown here for positions 220–280. On day 6, a 4–amino acid deletion was noted at positions 245–248; this deletion was present in all isolates collected after that day.

SmithKline) in a 96-well plate format. A/Wisconsin/67/2005 (H3N2) virus was used as control. The total protein for each preparation was determined by bicinchoninic acid assay after the manufacturer’s instructions (Invitrogen). The 50% inhibitory concentration (IC50) for each drug was determined by regression analysis (Prism, version 5.0b; GraphPad Software).

Virus replication and antiviral susceptibility. A high-throughput cell-based assay was used to determine the 50% effective concentration (EC50) as described elsewhere [35]. Assays were performed to test sensitivity to amantadine (Sigma), oseltamivir, and zanamivir. A/Wisconsin/67/2005 (H3N2) virus was used as control.

Plaque reduction assay. Madin-Darby canine kidney (MDCK) cells were inoculated with 100 plaque-forming units (PFUs) of virus per well. A 10-fold dilution of 0.0001–100 μmol/L oseltamivir tartrate solution (Hoffman–La Roche) or zanamivir solution (GlaxoSmithKline) was added to each well of a 6-well plate, with 1 well containing no drug. Plaques were counted after 48 h, and plaque reduction assays were performed in triplicate.

Viral replication assay. MDCK cells were infected with each viral isolate at a multiplicity of infection of 0.01. Plates were incubated at 37°C for 1 h. Cells were washed and incubated after the addition of 3 mL of Dulbecco’s modified Eagle medium containing 1 μg/mL tosyl phenylalanyl chloromethyl ketone–treated trypsin. Every 12 h for a 72-h period, 500 μL of supernatant was collected. Virus titers for each supernatant were determined in triplicate using a standard plaque assay technique [36]; mean titers were recorded (in PFUs per milliliter).

Ferret contact transmission. Sixteen seronegative 4-month-old male ferrets were housed in pairs, in separate cages with individual air flows. Two pairs (4 ferrets) were inoculated intranasally with 10^6 PFUs of influenza virus isolated from the patient at initial sampling, and 2 pairs were inoculated with 10^7 PFUs of the virus isolated from the patient 8 days after diagnosis. After 48 h, 2 uninoculated ferrets were placed in each cage with inoculated ferrets. Daily weights and temperatures were recorded, and nasal wash samples were obtained. Nasal wash sample titers were determined by standard plaque assay technique, and real-time RT-PCR was performed, as described elsewhere [37]. Amplification and sequencing of the NA gene was performed on each viral isolate recovered to determine whether the NA gene sequence remained stable after transmission and infection. Animal experiments were performed according to National Institutes of Health Institutional Animal Care and Use Committee–approved protocols and guidelines.

RESULTS

Identification and sequencing of viral isolates. Viruses isolated from patient nasal wash samples collected on days 0 (initial diagnosis), 2, 4, 6, 8, 10, and 12 were characterized. NA genes were amplified, and direct sequencing revealed that on days 0, 2, and 4 the NA gene was similar to the wild-type sequence of NA genes found in other A/Wisconsin/67/2005-like H3N2 viruses circulating during the 2007–2008 season. The virus isolated on day 6 and all subsequent isolates had a 12-nucleotide/4–amino acid in-frame deletion in the NA gene, corresponding to amino acid positions 245–248 (Figure 1). No other coding changes in the NA gene were noted, and no other known NAI resistance mutations were observed. The hemagglutinin genes of all viral isolates were also sequenced and showed no coding changes.

To determine whether this deletion was present at the time of diagnosis, a 600-bp region spanning the area of deletion of the NA gene was amplified from the nasal wash specimen collected on day 0. Sequencing of 50 clones did not reveal any deletions at amino acid positions 245–248.

Antiviral sensitivity. The IC50 values for the NAIs were
Table 1. Neuraminidase (NA) Activity and Antiviral Sensitivity

<table>
<thead>
<tr>
<th>Assay</th>
<th>A/Wisconsin/67/2005</th>
<th>Day 0</th>
<th>Day 8*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA activity, μU/μg total protein</td>
<td>85.3</td>
<td>6.0</td>
<td>1.4</td>
</tr>
<tr>
<td>NA inhibition assay, IC50, nmol/L (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>0.29 (0.27–0.32)</td>
<td>0.18</td>
<td>16.37</td>
</tr>
<tr>
<td>Zanamivir</td>
<td>3.07 (2.80–3.36)</td>
<td>4.00</td>
<td>12.70</td>
</tr>
<tr>
<td>High-throughput assay, EC50 (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amantadine, μmol/L</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Oseltamivir, nmol/L</td>
<td>0.37 (0.34–0.42)</td>
<td>0.21</td>
<td>442.60</td>
</tr>
<tr>
<td>Zanamivir, nmol/L</td>
<td>6.16 (5.79–6.57)</td>
<td>18.85</td>
<td>131.40</td>
</tr>
<tr>
<td>Plaque reduction assay, IC50 (95% CI), nmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>…</td>
<td>0.38</td>
<td>49.70</td>
</tr>
<tr>
<td>Zanamivir</td>
<td>…</td>
<td>15.58</td>
<td>224.50</td>
</tr>
</tbody>
</table>

NOTE. CI, confidence interval; EC50, 50% effective concentration; IC50, 50% inhibitory concentration.

* The day 8 isolates shows a deletion in NA of amino acid positions 245-248.

determined using an NA activity assay. The IC50 of oseltamivir for the virus containing the deletion isolated from the patient on day 8 (16.37 nmol/L; 95% confidence interval [CI], 12.72–21.07) showed a significant increase of >90-fold, compared with the day 0 wild-type virus (0.18 nmol/L; 95% CI, 0.18–0.19 nmol/L). A 3-fold increase in the IC50 of zanamivir was also noted when the day 8 virus was compared with the wild-type day 0 virus, with an increase from 4.0 (95% CI, 3.60–4.44) to 12.70 (95% CI, 12.08–13.37), respectively (Table 1). In addition to reduced sensitivity to antivirals, NA enzymatic activity associated with the day 8 virus was less than that for the day 0 isolate. Even though the day 0 isolate had a lower level of NA activity (measured per microgram) than A/Wisconsin/67/2005, another circulating H3N2 virus, it retained sensitivity to oseltamivir and showed a very modest decrease in sensitivity to zanamivir.

A high-throughput tissue culture assay demonstrated that both day 0 and day 8 virus isolates were resistant to amantadine, with a 50% effective concentration (EC50) of >200 μmol/L. The EC50 of oseltamivir for the wild-type virus isolated from day 0 was >2000-fold lower than that for the deletion-containing virus isolated on day 8. The EC50 of zanamivir was also increased 7-fold in the virus containing the deletion, compared with the wild-type day 0 virus, and 21-fold higher than that for the control virus (Table 1). Standard plaque reduction assays had results similar to those of the high-throughput assay, demonstrating a significantly higher IC50 for oseltamivir in the virus containing the deletion, with a >49-fold increase, and a 14-fold increase for zanamivir (Table 1).

The greater resistance demonstrated in these replication-dependent assays is likely to reflect not only the amplification of virus required for readout but also a reduced amount of NA in each virion. A comparison of the NA activity (per microgram total protein) in the virus preparations showed that the day 8 isolate had ~3 times less NA than the day 0 isolate (1.4 and 6.0 μU/μg, respectively). Interestingly, these isolates had substantially less NA activity than the A/Wisconsin/67/200 isolate (85.3 μU/μg) used as a control.

Viral fitness in cell culture. The virus isolated on day 8 containing the NA deletion showed no significant difference in viral replicative fitness in MDCK cells compared with the day 0 wild-type isolate. Both viruses reached peak titers (10⁵ PFUs/mL) at 36–48 h (Figure 2).

Ferret infection and contact transmission. All ferrets in...
transasally inoculated with either wild-type and NA mutant virus developed similar mild illness associated with significant nasal shedding of influenza A virus. Virus was detectable 48 h after inoculation by culture and RT-PCR of the nasal wash samples from all 4 ferrets in each group. No significant difference in detectable levels of virus was noted between the ferrets inoculated with the virus isolated on day 0 and those inoculated with the day 8 virus containing the NA deletion (Figure 3).

Three (75%) of 4 ferrets in each group that were not inoculated but were placed in cages with the inoculated ferrets were found to have detectable levels of virus in nasal wash fluid 48 h after contact. No significant difference in viral titers was observed between the ferrets placed in contact with the day 0 virus– versus the day 8 virus–inoculated ferrets (Figure 3).

**DISCUSSION**

We describe the isolation and characterization of an influenza A/H3N2 virus isolated containing a 4-amino acid deletion in the NA 5 days after initiation of oseltamivir therapy. This deletion was recently reported in another immunocompromised patient; however, that patient had an unusually long duration of illness (nearly 9 months), with the resistant strain isolated only after 3 months of continuous oseltamivir therapy. That patient had received multiple antiviral drugs, and the deletion occurred in the presence of other NA mutations previously associated with NAI resistance, including E119V and I222V [31]. In that report, it was hypothesized that the deletion may have led to some decrease in viral fitness, because it appeared only after months of continuous antiviral selective pressure. A reverse genetics–produced virus containing the deletion was studied in cell culture, but no conclusive evidence was obtained concerning the effect of this deletion on viral fitness [32].

In the present case, the NA deletion appeared rapidly in an immunocompromised adult who had 2 weeks of viral shedding and illness. In this instance, the duration of NA-selective pressure before the appearance of the resistance mutation was much shorter than in the prior case; we note that the recommended course of oseltamivir for influenza treatment is 5 days, but the drug is often given longer to immunocompromised patients. Thus, these findings demonstrate that NAI resistance can occur within the standard duration of administration, apparently in response to antiviral pressure. Although we cannot rule out the possibility that this deletion existed in a small subpopulation of the virus when the host became infected, its absence in the 50 NA clones sequenced from the original clinical specimen suggests that if this deletion was present, it was only a minor component. Given the available evidence, it appears likely that the deletion was rapidly selected for due to treatment with oseltamivir, because by day 6 of treatment the deletion-containing virus was the dominant viral genotype. Unlike the other reported instance of this deletion, this isolate did not bear other mutations commonly associated with NAI resistance.

There is no firm definition of a resistant IC\textsubscript{50} for NAIs, but a change in IC\textsubscript{50} of \(\geq 10\)-fold in a single virus before and after treatment is commonly considered the hallmark of resistance [38]. As was shown in the earlier characterization of a virus with this deletion [32], we found that the current isolate had a \(>10\)-fold increase in IC\textsubscript{50} for oseltamivir. These data, along with the apparent lack of clinical efficacy of the NAI, indicate that this deletion causes clinically significant resistance to oseltamivir. Also of interest is the reduction of sensitivity to zanamivir, primarily in cell culture and to a lesser degree in NA activity assays. This change may or may not correlate with clinically significant resistance; further studies are needed.

Characterization of the viral isolate containing this naturally occurring deletion revealed no differences in replicative fitness or transmissibility when the mutant was compared with the deletion-free isolate. No effect was seen on the ability of the virus to grow in cell culture, as judged by similar peak titers and growth curves. More compelling than the cell culture findings, we also observed that the duration and magnitude of shedding were similar in ferrets infected with either isolate. Thus, in standard models of influenza replication and trans-
mission, our data suggest that this deletion had no detrimental effect on viral fitness.

Although the factors underlying influenza evolution and selection are incompletely understood, transmissibility is probably a very important factor in determining whether a multidrug-resistant virus could successfully establish itself as a dominant strain in humans. The recent, unexpected emergence of an NAI-resistant seasonal A/H1N1 virus containing the NA H275Y mutation, with no apparent detriment to its transmissibility, is ample evidence that NAI resistance and evolutionary fitness need not be mutually exclusive.

The independent emergence of this NAI resistance-conferring deletion in 2 instances and the good viral fitness and efficient transmission of the isolate in a mammalian host raises the possibility that an H3N2 virus containing this 4-amino acid deletion may become more common, as did H275Y-containing H1N1 isolates. With clinically significant resistance to the adamantanes and oseltamivir and decreased sensitivity to zanamivir, this is a disconcerting possibility. That both occasions involved immunocompromised patients with prolonged illness due to influenza suggests that careful attention to diagnosis, treatment, and prevention of spread is warranted. Additionally, the value of rigorous surveillance in identifying and tracking the emergence of similar mutations cannot be underestimated. Such efforts could obviously be helpful in containing the emergence of resistant viruses, but they would also inform our understanding of the evolution of influenza and help us to assess whether immunocompromised persons are an important source for the emergence of antiviral resistance.

In conclusion, widespread emergence of antiviral resistance and identification of cases such as the one identified here suggest that resistance to NAs and the adamantanes is likely to increase as influenza viruses continue to evolve. The rapid emergence of oseltamivir resistance in an already amantadine-resistant A/H3N2 virus highlights the difficulties we face in treating influenza, especially in patients who are susceptible to prolonged infection, such as immunocompromised individuals. The prompt emergence of this multidrug-resistant virus during clinical NAI therapy, along with the lack of apparent detriment to viral fitness and transmissibility, suggests that this resistance-conferring NA gene deletion as well as other resistance conferring mutations may not be isolated events and may become more widespread as use of NAI increases, especially in the immunocompromised or chronically ill. Future studies to evaluate influenza treatment and prevention strategies for immunocompromised individuals, will need to include careful consideration of the rapid development of drug resistance during antiviral treatment.

Acknowledgments

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References

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