Characterization of Multidrug-Resistant Influenza A/H3N2 Viruses Shed during 1 Year by an Immunocompromised Child

Mariana Baz,1 Yacine Abed,1 Jane McDonald,2 and Guy Boivin1
1Research Center in Infectious Diseases of the Centre Hospitalier Universitaire de Québec and Laval University, Québec City, and 2Montréal Children’s Hospital, McGill Health Center and University, Montréal, Canada

(See the editorial commentary by Kaiser on pages 1562–4)

Background. Development of influenza drug resistance is an important problem in immunocompromised children that could result in treatment failure and viral transmission to others.

Methods. A total of 17 influenza A/H3N2 isolates were recovered over a period of 1 year from an immunocompromised child who was initially treated with oseltamivir and then with amantadine and zanamivir for viral pneumonitis. Drug susceptibility phenotypes to oseltamivir, zanamivir, and peramivir were evaluated by neuraminidase (NA) inhibition assays, and sequence analysis of key viral genes (i.e., M2, NA, and hemagglutinin [HA]) was performed. The impact of NA mutations identified in oseltamivir-resistant isolates was analyzed using recombinant NA proteins.

Results. An influenza A variant with NA mutations E59G, E119V, and I222V was first detected after 38 days of oseltamivir treatment. In an NA inhibition assay, this variant was 274 times more resistant to oseltamivir than the original isolate but was susceptible to zanamivir. The I222V substitution enhanced the level of oseltamivir resistance that was primarily conferred by the E119V mutation in recombinant NA proteins. Remarkably, the E119V mutation persisted for 8 months after cessation of oseltamivir. Amantadine therapy led to rapid emergence of the M2 mutation S31N, which is known to confer amantadine resistance. The patient shed the virus intermittently while receiving nebulized zanamivir therapy despite the absence of a resistance phenotype, which could be the result of nonoptimal drug delivery and impaired host immunity.

Conclusions. This study highlights the potential for emergence and persistence of multidrug-resistant influenza isolates in immunocompromised subjects even after cessation of treatment, reinforcing the need for development of new anti-influenza compounds.

Influenza A viruses cause significant morbidity and mortality in humans. Such infections are particularly severe in transplant recipients, in whom they have been associated with a high rate of pulmonary complications and an increased risk of graft dysfunction and rejection [1]. Influenza viruses may also predispose immunocompromised subjects to lethal infection in association with other pathogens, such as Aspergillus fumigatus [2]. In addition, transplant recipients may experience prolonged shedding of influenza viruses, oftentimes despite antiviral therapy [3–5].

Two classes of antiviral agents have been used to control influenza infections, including the M2 ion channel blockers and the neuraminidase inhibitors (NAIs). The use of M2 blockers (amantadine and rimantadine), which are effective against influenza A viruses only, is associated with the rapid emergence of drug resistance mutations at codons 26, 27, 30, 31, or 34 of the M2 protein [6, 7]. In addition, natural resistance to M2 inhibitors has been increasing; 92.3% of influenza A/H3N2 viruses isolated in the United States during the period 2005–2006 harbored the M2 S31N resistance mutation [8]. Many recent human isolates of highly virulent A/H5N1 influenza viruses also express primary resistance to these drugs [9–11]. Amantadine-resistant variants are genetically stable, are pathogenic, and can be transmitted from person to person [12, 13].
Inhaled zanamivir and the orally bioavailable drug oseltamivir are the only 2 currently approved NAIs [14]. Other orally bioavailable NAIs, such as peramivir [15] and A-315675 [16], are in various stages of development. Thus far, there have been only a few reports of influenza B viruses with reduced susceptibility to zanamivir [17, 18]. Oseltamivir-resistant strains have been infrequently detected in clinical trials, with an estimated frequency of 0.4%–1% and 4%–8% in adults and children, respectively, treated in an ambulatory setting [19, 20]. However, a higher incidence (up to 18%) of oseltamivir resistance has been recently reported in hospitalized Japanese children [21]. Oseltamivir-resistant strains have been shown to contain neuraminidase (NA) substitutions at residues 119, 292, and 294 in influenza A/H3N2 viruses [21–23] and at residue H274Y in influenza A/H1N1 and influenza A/H5N1 viruses [4, 22, 24, 25]. Emergence of multidrug-resistant influenza viruses has also been recently reported in immunocompromised patients [4, 23, 26].

In this study, we report the molecular evolution of influenza A/H3N2 viruses associated with multidrug resistance over 1 year in an immunocompromised child.

**MATERIALS AND METHODS**

**Patient.** The child received a diagnosis of severe combined immunodeficiency disease (SCID) at 4 months of age (in May 2002) and rapidly received an allogeneic haploidentical stem cell transplant from her father. Because of nongraftment, she received a second stem cell transplant with conditioning from her father in November 2002, but she remained pancytopenic. She required reinfusion of CD34+ cells in January 2003, resulting in better engraftment. She was treated with steroids and mycophenolate mofetil because of cutaneous graft-versus-host disease (from June 2003 to June 2005) and then with steroids only. In terms of infectious disease complications, she had mild chronic diarrhea due to rotavirus from June 2002 until February 2003. She also developed a chronic lower respiratory tract infection due to human parainfluenza type 3 from the time of SCID diagnosis until just after the more-successful engraftment (March 2003) that was treated with inhaled ribavirin during a period of 10 months.

Infection with influenza A was diagnosed in April 2005 and progressed to a chronic pneumonia of the lingula. She was initially treated with the recommended dose of oseltamivir (2 mg/kg/dose, 2 times daily [i.e., 24 mg, 2 times daily]) for 2 weeks, with some improvement. However, clinical symptoms reappeared and viral culture results became positive shortly thereafter, which led to long-term administration of oseltamivir (10–20 mg 2 times daily via a nebulizer). The patient also received liposomal amphotericin B and caspofungin followed by voriconazole suppressive therapy for aspergillosis. The patient and her family were vaccinated with the inactivated influenza vaccine on 1 November 2005. She received another infusion of stem cells in January 2006 because of poor immune reconstitution and persistent influenza virus infection. As of July 2006, the patient is still lymphopenic (800 × 10⁹ cells/L, mostly T cells) and has chronic skin graft-versus-host disease, which is treated with steroids (prednisone, 2.5 mg 2 times daily). She has mild chronic cough with no need for supplemental oxygen while receiving nebulized zanamivir. The results of her 3 most recent viral cultures (from June, July, and August 2006) have been negative for influenza.

**Viral isolates.** Nasopharyngeal aspirates or bronchoalveolar lavage samples were tested for the presence of influenza viruses by viral culture on Madin-Darby canine kidney cells. A maximum of 3 passages were performed prior to phenotypic and genotypic analyses.

**Sequence analyses of virus isolates.** RNA was isolated from cell culture supernatants using the QIAamp Viral RNA kit (Qiagen), and complementary DNA (cDNA) was synthesized using random hexamer primers (Amersham Pharmacia Biotech) and the SuperScript II reverse transcriptase ( GibCO-BRL). Viral cDNA was amplified by PCR using the Pfu Turbo Polymerase (Stratagene) and NA-, hemagglutinin (HA)–, and M2-specific primers in standard conditions. Nucleotide sequences of PCR products were determined using an automated DNA sequencer (ABI Prism 377 DNA sequencer; Applied Biosystems).

**Cloning of PCR products.** To estimate the relative proportions of wild-type (WT) and mutant genes in some clinical isolates, PCR products were cloned by blunt-end ligation into the vector pBluescript II KS+ (Stratagene), and at least 8 recombinant plasmids were selected before sequence analysis. Selected influenza viruses were also plaque-purified once before sequence analysis.

**Susceptibility assays.** Madin-Darby canine kidney cells were inoculated with 80–100 plaque-forming units of virus per well in 6-well plates for conventional plaque reduction assays [27]. NA inhibition was assayed with 800–1200 fluorescence units of virus in presence of serial halff-log dilutions (from 0.038 to 10540 nmol/L) of zanamivir, oseltamivir carboxylate (both synthesized at GlaxoSmithKline, Stevenage, United Kingdom), and peramivir/BCX-1812 (BioCryst). The methylumbelliferyl-N-acetylneuraminic acid (Sigma) was used as a fluorescent substrate [28].

**Hemagglutination and elution assays.** Hemagglutination assays were performed in U-bottom microtiter plates using 50 μL of a 1% suspension of human RBCs and 50 μL of serial 2-fold dilutions of virus in phosphate-buffered saline. Plates were

---

1556 • CID 2006:43 (15 December) • Baz et al.

---

1556 • CID 2006:43 (15 December) • Baz et al.
Figure 1. Phenotypic and genotypic characterization of influenza A/H3N2 viruses recovered from an immunocompromised child treated with oseltamivir, amantadine, and zanamivir. All influenza A cultures were performed using nasopharyngeal aspirates, with the exception of 2 bronchoalveolar lavage specimens that were obtained on 10 June 2005 and 16 August 2005. NA, not applicable because of negative influenza culture result; ND, not determined. *Sample positive for influenza A virus by antigen detection test.
Table 1. Detailed genotypic and phenotypic evaluation of selected influenza A isolates recovered from an immunocompromised child.

<table>
<thead>
<tr>
<th>Clinical sample</th>
<th>NA substitution(s)</th>
<th>HA substitution(s)</th>
<th>M2 substitution(s)</th>
<th>Oseltamivir IC₅₀ (fold change), nmol/L</th>
<th>Zanamivir IC₅₀ (fold change), nmol/L</th>
<th>Peramivir IC₅₀ (fold change), nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>2 ± 0.6</td>
<td>3.6 ± 0.5</td>
<td>0.23 ± 0.6</td>
</tr>
<tr>
<td>6</td>
<td>E119V</td>
<td>N189K, G479E</td>
<td>...</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>7</td>
<td>E59G, E119V, I222V</td>
<td>N189K, T440S, G479E</td>
<td>...</td>
<td>548 ± 187 (274)</td>
<td>6.21 ± 0.34 (1.7)</td>
<td>0.8 ± 0.08 (3.5)</td>
</tr>
<tr>
<td>8</td>
<td>E119V</td>
<td>...</td>
<td>...</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>11</td>
<td>E119V</td>
<td>...</td>
<td>S31N</td>
<td>2012 ± 338.84 (1006)</td>
<td>5.29 ± 2.43 (1.5)</td>
<td>1.71 ± 0.58 (7.4)</td>
</tr>
<tr>
<td>13</td>
<td>E119V, I222V</td>
<td>...</td>
<td>S31N</td>
<td>386 ± 68.06 (193)</td>
<td>9.87 ± 1.05 (2.7)</td>
<td>1.62 ± 0.06 (7)</td>
</tr>
<tr>
<td>20</td>
<td>E119V/E, N146K, S219T, De1245–248</td>
<td>...</td>
<td>S31N</td>
<td>109 ± 20 (54)</td>
<td>10.1 ± 2.4 (2.8)</td>
<td>0.33 ± 0.1 (1.43)</td>
</tr>
<tr>
<td>21</td>
<td>N146K, S219T, A272V, De1245–248</td>
<td>...</td>
<td>S31N</td>
<td>926 ± 66 (463)</td>
<td>7.9 ± 2.69 (2.2)</td>
<td>0.42 ± 0.03 (1.8)</td>
</tr>
<tr>
<td>22</td>
<td>E119V, I222V, V194I, T242I</td>
<td>H56Y, IS8, T128N, S138A, N144K, V2231, M346L, N375D, L409I, K453R</td>
<td>S31N</td>
<td>210 ± 23 (105)</td>
<td>4.7 ± 0.4 (1.3)</td>
<td>0.53 ± 0.02 (2.3)</td>
</tr>
<tr>
<td>24</td>
<td>Y50F, N71S, E119V, D309N</td>
<td>H56Y, IS8, T128N, S138A, N144K, H156N, K158N, V2231, M346L, N375D, L409I, K453R</td>
<td>S31N</td>
<td>0.88 ± 0.32 (0.44)</td>
<td>0.52 ± 0.13 (0.16)</td>
<td>0.12 ± 0.01 (0.52)</td>
</tr>
</tbody>
</table>

NOTE. HA, hemagglutinin; NA, neuraminidase.

a Data are average IC₅₀ values of at least 3 independent experiments ± SD. Where indicated, fold increases in IC₅₀ value compared with that of the wild-type virus are included in parentheses.

b No values were obtained because of poor growth.

c No values were obtained because of insufficient NA activity for NA inhibition assays.

incubated for 1 h at 4°C. Hemagglutination-elution assays were performed in the absence or presence of 1 μmol/L of zanamivir as described elsewhere [29].

Expression of recombinant NA proteins. The NA gene of an influenza A/Sydney/5/97 H3N2-like virus was amplified by PCR with primers containing the A/WSN/33 H1N1 NA non-coding regions and the SapI restriction site, followed by ligation into the empty pPOLI-SapI-Rb plasmid as previously described [30]. The resulting pPOLI-NA₅₃ plasmid was used for incorporation of some NA mutations identified in the patient’s clinical isolates using appropriate primers and the QuickChange Site-Directed Mutagenesis kit (Stratagene). For expression of recombinant NA proteins, 293T (human embryonic kidney) cells were cotransfected with 1 μg of each of the 4 expression plasmids (pCAGGS-PA, -PB1, -PB2, and -NP) of A/WSN/33 and the respective pPOLI-NA₅₃ plasmid. At 48 h after transfection, cells were treated with 0.02% ethylenediaminetetraacetic acid in phosphate-buffered saline and harvested. The cells were resuspended in phosphate-buffered saline containing 3.5 mmol/L CaCl₂, and stored at −80°C. Total proteins from transfected cells were quantified using the BCA protein assay (Pierce) and NA inhibition assays were performed as described above using an equivalent quantity of expressed proteins [31].

RESULTS

Characterization of influenza isolates. A total of 27 nasopharyngeal aspirates and 2 bronchoalveolar lavage samples were recovered from an immunocompromised child over 12 months. Among these samples, 17 influenza A isolates were obtained by cell culture (figure 1). Eleven clinical isolates selected from different periods were further subtyped by RT-PCR as A/H3N2 and then propagated in Madin–Darby canine kidney cells before phenotypic and genotypic analysis (table 1). Isolate 2, which was an A/California/7/2004-like strain, was recovered after 14 days of oseltamivir therapy. This isolate was susceptible to the drug in NA inhibition assay, with an IC₅₀ of 2.0 nmol/L. A subsequent virus (isolate 7), which was recovered from a sample of bronchoalveolar lavage fluid after 38 days of cumulative oseltamivir treatment, had an oseltamivir IC₅₀ of 548
nmol/L (274-fold higher than the IC\textsubscript{50} of isolate 2) but remained susceptible to zanamivir and peramivir. Sequence analysis of 8 cDNA clones of the HA and the NA genes of this isolate revealed 3 aa substitutions in the NA gene (E59G [87.5% of clones], E119V [100%], and I222V [100%]) and 3 aa substitutions in the HA gene not found in subsequent isolates (table 1). Amantadine treatment was added to oseltamivir after 89 days of therapy because of persistent viral shedding. Sequence analysis of the M2 gene revealed the emergence of the S31N aa substitution after 5 days of amantadine treatment (isolate 11). At this point, only the E119V NA mutation was detected, but no NAI assay could be performed because of poor enzymatic activity. Isolate 13, recovered after 105 days of cumulative oseltamivir treatment, had an IC\textsubscript{50} of 2012 nmol/L (1006-fold change compared with WT) for oseltamivir, whereas IC\textsubscript{50} values for zanamivir and peramivir showed modest variations (<10-fold change compared with WT). At this point, retrospective genotypic analysis revealed the presence of the S31N aa substitution after 5 days of amantadine treatment (isolate 11). The results of subsequent viral cultures were negative for many weeks, and the decision was made to stop therapy on 15 October 2005. However, viral shedding eventually resumed and persisted despite reintroduction of amantadine and zanamivir on 1 November 2005. Phenotypic analysis of the clinical isolate from 4 November 2005 (isolate 20), recovered 76 days after oseltamivir discontinuation, still showed an oseltamivir-resistant virus with an IC\textsubscript{50} of 386 nmol/L (a 193-fold increase) with no significant changes in zanamivir and peramivir susceptibilities (table 1). No mutations in the HA gene were detected, but the analysis of the NA gene revealed a mixed population of WT virus and E119V mutants, as well as 2 new NA mutations (N146K and S219T) and a deletion of 4 aa. The E119V mutation could not be detected in isolate 21 and in 8 plaque-purified viral strains; the oseltamivir IC\textsubscript{50} value of this isolate was 109 nmol/L, which was only 54 times higher than that of the parental isolate (table 1).

Isolate 22 recovered after 90 days of cumulative zanamivir treatment and 105 days after cessation of oseltamivir still showed an increased IC\textsubscript{50} for oseltamivir (926 nmol/L, a 463-fold increase) and no significant change for the other NAIs in NA inhibition assays. Also, susceptibility testing performed with a plaque reduction assay did not reveal a significant difference in zanamivir IC\textsubscript{50} between this isolate and the initial one (133 nmol/L and 156 nmol/L, respectively) whereas an oseltamivir-resistant isolate (E119V) had a >100-fold increase in oseltamivir IC\textsubscript{50} value compared with the susceptible isolate. Similarly, isolate 22 and the parental one did not elute human RBCs after an overnight incubation at 37°C in the presence or absence of zanamivir. The analysis of plaque-purified viral strains revealed the following NA mutations: E119V (100%), I222V (70%), V194I (30%), and T242I (100%); it also revealed 10 new HA substitutions (table 1). None of these substitutions were part of the HA receptor binding site except S138A.

Isolate 24, recovered after 139 days of cumulative zanamivir therapy, had an IC\textsubscript{50} of 210 nmol/L (a 105-fold increase) for oseltamivir without significant change in susceptibility for zanamivir and peramivir. Genotypic analysis of 10 randomly selected individual plaques revealed the presence of the E119V mutation (100%), as well as the new NA mutations Y50F (100%), N71S (100%), and D309N (75%), whereas 2 new aa substitutions were found in the HA protein. The last positive clinical isolate studied (isolate 29), recovered after 212 days of cumulative zanamivir therapy and 233 days after the end of oseltamivir therapy, was now susceptible to oseltamivir (IC\textsubscript{50} 0.88 nmol/L) as well as to zanamivir and peramivir. Genotypic analysis of 10 randomly selected individual plaques revealed 3 new NA substitutions (I215V [100%], N329T [100%], and S372L [100%]), in addition to the D309N change present in the previous isolate. Of note, the E119V mutation was no longer present. The HA gene contained 5 new aa substitutions not found in the previous isolate. Finally, the M2 S31N mutation associated with amantadine resistance remained present more than 133 days after drug cessation.

**Characterization of recombinant NA mutant proteins.**

The NA activity of the recombinant NA mutant proteins expressed in 293 T cells varied from 60% (E119V) to 123% (I222V), compared with that of the WT NA protein (table 2). The E119V mutant protein was 131 times more resistant to oseltamivir than the WT, whereas the I222V and E59G proteins had respectively modest and no increase in oseltamivir IC\textsubscript{50} values, respectively, compared with the WT. Interestingly, the presence of the E119V and I222V substitutions had synergistic effects on the oseltamivir resistance phenotype (a 293-fold change in IC\textsubscript{50}), whereas the NA protein with the 3 changes (E119V, I222V, and E59G) had lower oseltamivir IC\textsubscript{50} values.

<table>
<thead>
<tr>
<th>NA proteins</th>
<th>Oseltamivir carboxylate IC\textsubscript{50} (nmol/L ± SD)</th>
<th>Fold change</th>
<th>NA activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.45 ± 0.21</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>E119V</td>
<td>452 ± 102</td>
<td>131</td>
<td>60</td>
</tr>
<tr>
<td>I222V</td>
<td>6.65 ± 2.05</td>
<td>1.9</td>
<td>123</td>
</tr>
<tr>
<td>E119V and I222V</td>
<td>1011 ± 85</td>
<td>293</td>
<td>72</td>
</tr>
<tr>
<td>E59G</td>
<td>2.93 ± 3.3</td>
<td>0.84</td>
<td>110</td>
</tr>
<tr>
<td>E59G, E119V, and I222V</td>
<td>649 ± 3</td>
<td>188</td>
<td>84</td>
</tr>
</tbody>
</table>

* Data are average IC\textsubscript{50} values of at least 3 independent experiments ± SD.

**NOTE.** WT, wild type.
(188-fold) in agreement with the susceptibility profiles of isolates 13 and 7, respectively.

**DISCUSSION**

Antiviral therapy with either M2 blockers or NA inhibitors is thought to reduce the duration of viral shedding, the risk of progression to pneumonia, and, possibly, the mortality associated with influenza in immunocompromised hosts, although no clinical trials have been performed in these patients [1, 3, 32]. However, some of these individuals may develop sustained viral replication despite antiviral treatment, which is a risk factor for emergence of drug resistance [4, 26].

In this study, we reported the recovery of many dual NA inhibitor– and M2 blocker–resistant mutants in an immunocompromised child who shed influenza A/H3N2 viruses for 1 year. Virus isolates from our immunocompromised patient had many changes in the NA gene, including substitutions and deletions. After ∼1 month of cumulative oseltamivir treatment, the patient shed a virus with the previously described E119V mutation, which is the most frequently reported A/H3N2 mutation conferring oseltamivir resistance. Also, as reported, this mutant remained susceptible to zanamivir and to the investigational drug peramivir [23, 26]. Importantly, this framework mutation persisted >7 months after cessation of oseltamivir treatment, indicating that NA mutation can be pathogenic and stable in the absence of drug pressure in immunocompromised subjects. As previously reported [21, 23], quasi species of resistant and susceptible variants were present in our patient at some points. Indeed, a mixture of 2 variants at codon 119 was found in isolate 20, whereas the E119V mutation was transiently undetectable in 8 clones of isolate 21. This suggests that the presence of mixed viral populations may be underestimated with actual detection methods based on PCR and sequencing. Some oseltamivir-resistant isolates also contained other aa substitutions, such as E59G and I222V. Residue 59 is not a framework or catalytic residue within the viral protein [33] and is not known to confer resistance to oseltamivir. In contrast, residue I222 is a highly conserved framework residue among all influenza A and B viruses [33]. The I222V substitution has been reported to contribute to oseltamivir resistance in an A/Texas/36/91 (H1N1) virus together with the H274Y mutation [34]. In that study, the single I222V mutant conferred a 2-fold change in oseltamivir susceptibility, whereas the double mutant (I222V and H274Y) was associated with a 1000-fold decrease in drug susceptibility. Our data that were obtained with recombinant mutant proteins also indicate a modest role in resistance for the single I222V mutation but a synergistic role when associated with the primary resistance mutation E119V. The E59G change could be a compensatory mutation, although this needs to be formally proven. The role of other unknown NA mutations in some of our patient’s isolates is uncertain although they do not seem to have contributed significantly to the drug resistance phenotypes in addition to the E119V mutation.

Because of the progression of respiratory symptoms and persistently positive viral culture results, the treatment of our patient was switched from oseltamivir to amantadine and zanamivir. After only 5 days of amantadine therapy, a virus with a S31N substitution in the M2 gene was isolated. This mutation is the most frequently reported amantadine resistance mutation in influenza A/H3N2 viruses [5–7, 23]. The S31N mutation persisted for >100 days after drug cessation. Recently, the S31N mutation has been frequently reported in both A/H3N2 and A/H5N1 isolates from untreated patients, highlighting the genetic stability and transmissibility of the M2 mutants [8–12].

Interestingly, our patient continued to shed influenza virus while receiving zanamivir treatment despite the absence of detectable resistant viruses by NA inhibition assays. It has been shown, however, that HA mutations can confer resistance to NAIs in vitro [24, 35], and we indeed found many HA mutations that occurred after 3 months of zanamivir. Although 2 of these mutations (V223I and S138A) are near or within the receptor binding site of the HA, our HA mutants exhibited no change in zanamivir susceptibility when tested by plaque-reduction or HA elution assays when compared with our patient’s initial isolate. We hypothesize that persistent viral shedding while receiving zanamivir therapy could be explained by low penetration of the drug in the respiratory tract when it is nebulized. Indeed, the recommended mode of administration of zanamivir by an inhaler was not possible in our young patient. Because our patient had been vaccinated at the beginning of November 2005, it is also possible that new HA mutations could have appeared through some immunologic pressure following the development of antibodies. Finally, since the last isolate recovered in April 2006 did not contain the E119V mutation and had an almost complete set of new NA and HA mutations, it is also possible that some recombination of preexisting viruses or a new infection occurred. Indeed, the final isolate had a lower HA nucleotide identity than did the initial isolate (98% vs. 99.6%) with the vaccine strain A/California/7/2004. Among the limitations of our study, we note the potential selection of mutations following in vitro passages, the unreliability of plaque reduction assays performed with Madin–Darby canine kidney cells, and the noncharacterization of HA mutations.

In conclusion, our study illustrates the rapid molecular evolution of influenza A viruses in immunocompromised patients and the potential for chronic viral infection with development of multidrug resistance. The long term persistence of viral mutations conferring drug resistance many months after cessation of oseltamivir and amantadine treatment and the difficulty encountered with zanamivir administration in severely ill patients

1560 • CID 2006:43 (15 December) • Baz et al.

Downloaded from https://academic.oup.com/cid/article-abstract/43/12/1555/278073 on 06 May 2018
reinforce the need to develop new antiviral compounds and innovative approaches [36].

Acknowledgments

Financial support. Canadian Institutes of Health Research (69559 to G.B.).

Potential conflicts of interest. All authors: no conflicts.

References