Prolonged Excretion of Amantadine-Resistant Influenza A Virus Quasi Species after Cessation of Antiviral Therapy in an Immunocompromised Patient

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Phenotypic and molecular studies were conducted to characterize multiple influenza A isolates recovered from an immunocompromised patient who died of viral and fungal pneumonitis. The recovery of amantadine-resistant isolates was correlated with the detection of 2 drug-resistant M2 variants (codons 27 and 31) in combination with a wild-type virus. The mutant viruses persisted within the viral population in variable proportions >1 month after cessation of antiviral therapy. These results confirm animal studies reported elsewhere regarding the genetic stability of influenza M2 mutants and their potential for transmission in humans.

Amantadine inhibits influenza A viruses at concentrations of <1 μg/mL by blocking the ion channel formed by the M2 protein and preventing viral uncoating. Resistance to amantadine is relatively common among viruses recovered from both immunocompetent [1, 2] and immunocompromised [3, 4] treated patients. Amantadine-resistant virus strains have also been found, although infrequently, in untreated patients as a result of virus transmission from treated subjects [1, 5] or of circulation of naturally occurring drug-resistant variants [6]. The molecular basis for amantadine and rimantadine resistance has been attributed to amino acid substitutions at 1 of 5 codons (26, 27, 30, 31, and 34) in the transmembrane domain of the M2 protein [3, 7]. We describe a case of fatal viral pneumonitis in an immunocompromised patient who shed amantadine-resistant influenza A viruses >1 month after cessation of antiviral therapy.

Case report. A 57-year-old man with chronic lymphoid leukemia was admitted to the hospital on 28 January 1999 for cough and fever that occurred a few weeks after he underwent chemotherapy. His chemotherapy regimen included cyclophosphamide, doxorubicin, vincristine, and prednisone. Analysis of chest radiographs revealed the presence of a diffuse interstitial pneumonitis. A nasopharyngeal aspirate recovered at admission was positive for respiratory syncytial virus (RSV) by use of EIA. Bronchoscopy performed a few days later revealed the presence of Pneumocystis carinii cysts, although alveolar lavage was negative for bacteria and viruses. The patient was treated with a course of trimethoprim-sulfamethoxazole for 1 month and with nebulized ribavirin for 3 days.

After initial improvement, the patient presented with an influenza-like illness during his hospitalization (nosocomial acquisition from an unknown source), and a nasopharyngeal aspirate specimen tested positive for influenza A virus by an immunofluorescence assay on 18 February 1999, which was confirmed by a positive result of culture of a sputum sample (sample 1) 4 days later. Amantadine (100 mg b.i.d.) was prescribed for 14 days starting on 18 February. After some clinical improvement, the patient was discharged on 3 March, but he was readmitted on 7 March for increased dyspnea and productive cough. A second bronchoscopy was performed on 8 March and the sample obtained was positive for influenza A by immunofluorescence. The patient was treated with a second course of amantadine (100 mg b.i.d.) from 8–17 March, and he was discharged on 17 March with improving respiratory symptoms and findings on chest radiographs.

On 15 April 1999, the patient was readmitted for a third time because of fever, persistent cough, hypoxemia, and deteriorating pulmonary infiltrates. Cultures of 2 sputum samples obtained at admission were both positive for influenza A (samples 2 and 3), adenovirus, and Aspergillus fumigatus. A third bronchoscopy performed on 20 April revealed positive cultures for influenza A (sample 4, bronchial secretions; sample 5, alveolar lavage), adenovirus, and A. fumigatus. An open biopsy of the left lung was performed a few days later because of the patient’s deteriorating condition, and the findings of histopathologic analysis were compatible with viral and fungal pneumonitis. The biopsy specimens were also positive for A. fumigatus by culture, influenza A by immunofluorescence, and RSV by use of EIA. Starting on 20–22 April, the patient was treated with trovafloxacin, amantadine, amphotericin B, and

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nebulized ribavirin with γ-globulins. The patient underwent intubation on 28 April 1999 and died on 2 May 1999. No autopsy was performed.

Influenza isolates were recovered by culture on Madin-Darby canine kidney cells from sputum or bronchoalveolar lavage samples. Viral typing and subtyping were performed by use of monoclonal antibodies (BioWhittaker) and reverse-transcriptase PCR (RT-PCR) for the hemagglutinin gene, respectively [8]. Susceptibility to amantadine, reported as the 50% inhibitory concentration (IC₅₀), was assessed by a plaque-reduction assay, which tested serial concentrations of the drug (0.025–200 μg/mL) in duplicate in 6-well plates [9]. Resistance to amantadine was defined as an IC₅₀ of ≥1 μg/mL [3].

Mutations in the influenza A M2 gene were assessed in 2 ways. First, sequence analysis of the RT-PCR–amplified M2 region of RNA segment 7 was performed as described elsewhere [3]. In addition, each amplified product was digested with 2–5 U of the restriction enzymes SspI, Itali, MwoI, and AcI to look for the presence of wild-type or mutant sequences at codons 31, 30, 27, and 26, respectively [3]. Digested products were run on a 1.5% agarose gel stained with ethidium bromide. The ratio of different variants was estimated by measuring the intensity of specific bands on gel with the AlphaImager 2000 (Alpha Innotech).

Results. Five influenza A (H3N2) viruses recovered from sputum or bronchoalveolar lavage samples were available for phenotypic and genotypic analyses (table 1). The first virus, which was recovered on the fourth day of amantadine therapy, was susceptible to the drug, with an IC₅₀ of 0.1 μg/mL. Subsequent viruses (numbers 2–5), which were recovered 28–33 days after cessation of a 24-day cumulative amantadine treatment, had IC₅₀ values 400–1000-fold higher than the value for the first isolate (IC₅₀ range, 41.3–100.3 μg/mL). Sequence analysis of the M2 RT-PCR-amplified product revealed a mutation at codon 31 (Ser→Asn) in viruses from samples 2–5 and a second mutation at codon 27 (Val→Ala) in viruses recovered from samples 2–4. Restriction enzyme analysis of the amplified products showed variable mixtures of mutant and wild-type products among different IC₅₀ values at codons 27 and 31 in the last 4 samples (table 1; figure 1). The genetic relatedness between the 5 isolates was confirmed by virtue of identical sequences for the hypervariable hemagglutinin-1 region (data not shown).

Discussion. The case we report is exceptional in many ways. First, the patient we describe, who had chronic lymphoid leukemia, developed pneumonitis due to multiple viral (influenza A, RSV, and adenovirus) and fungal (Pneumocystis carinii and Aspergillus fumigatus) pathogens. Although it is difficult to determine the impact of each pathogen on the patient’s progression to respiratory failure, the role of the influenza A virus is unequivocal, as confirmed by its presence in 2 of the last 3 bronchoalveolar lavage samples and in the findings of an examination of an open biopsy specimen of the left lung.

Second, our case illustrates the extraordinary heterogeneity of influenza A virus populations that can coexist in immunocompromised patients. A few cases of immunodeficient subjects infected with dual M2 gene mutations have been described elsewhere [3, 4]. Our study expands these previous results by showing the simultaneous presence of 2 mutants at codons 31 and 27, each mixed with a wild-type virus in many isolates recovered from our patient (figure 1). Of note, 2 viruses (from samples 4 and 5), which were recovered on the same day from bronchial secretions and alveolar lavage specimens, contained different viral populations: a double (codons 27 and 31) and a single (codon 31) mutant, respectively (table 1). As reported elsewhere [7], mutations at codon 31 of the M2 protein are the most frequently reported mutations in amantadine- and rimantadine-resistant isolates.

At this time, however, insufficient data exist on the impact of different mutations on the drug phenotype and on the pathogenicity or transmissibility of various mutants. Our study shows that mutation at codon 31 (Ser→Asn) was associated with a resistant phenotype (isolate 5; table 1) and that the combination of the 2 mutants (codons 31 and 27) mixed with wild-type virus (isolates 2–4) resulted in even higher IC₅₀ values,

Table 1. Phenotypic and molecular analyses of influenza A virus isolates recovered from an immunocompromised patient.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Date specimen was obtained</th>
<th>Specimen type</th>
<th>Duration of amantadine treatment or interruption, days</th>
<th>IC₅₀ of amantadine, μg/mL</th>
<th>M2 codons (%) of mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22 Feb 1999</td>
<td>Sputum</td>
<td>Receiving treatment 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>15 Apr 1999</td>
<td>Sputum</td>
<td>Not receiving treatment 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15 Apr 1999</td>
<td>Sputum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>20 Apr 1999</td>
<td>Bronchial secretion</td>
<td>0</td>
<td>33</td>
<td>100.3</td>
</tr>
<tr>
<td>5</td>
<td>20 Apr 1999</td>
<td>Alveolar lavage</td>
<td></td>
<td>33</td>
<td>41.3</td>
</tr>
</tbody>
</table>

**NOTE.** A control strain (A/Sydney/5/97 [H3N2]) had a 50% inhibitory concentration (IC₅₀) of 0.3 μg/mL (range, 0.2–0.4 μg/mL).

* Results are based on the mean of the values for 2 experiments.
Figure 1. PCR restriction analysis of the M2 gene in multiple influenza A isolates (samples 1–5; table 1) recovered from an immunocompromised patient. PCR products were digested with enzyme SspI (left) and MwoI (right) to determine the presence of mutations at codons 31 and 27, respectively [3]. Sample 1 is a wild-type virus for both codons, and samples 2–5 contain variable proportions of mutations at 1–2 codons.

which were ~1000-fold higher than the IC50 of the wild-type isolate recovered early in the course of therapy.

The most interesting finding of our study is related to the prolonged excretion of influenza A mutant viruses after cessation of amantadine therapy. Although continuous shedding of resistant viruses after removal of antiviral pressure has been reported elsewhere [3, 4], the persistence of such viral mutants >1 month after the end of therapy in our patient is, to our knowledge, the longest described. Such in vivo results confirm the findings of animal studies that have demonstrated the genetic stability of some amantadine-resistant viruses [10]. Resistant viruses have also been shown to replicate as well as wild-type viruses in some animal experimental models with similar pathogenic potential [10, 11]. However, it is not known for how long such mutants can be perpetuated in nature in the absence of drugs.

A recent study has reported the circulation of drug-resistant viral mutants (mainly Ser31Asn) in the absence of drugs, although it was a rare event [6]. Besides the fact that the presence of amantadine-resistant viruses may have contributed to our patient’s death, the prolonged shedding of such viruses has also important public health implications, because some studies have shown that they could be efficiently transmitted [1, 5]. Future studies are needed to evaluate the efficacy of the newly available neuraminidase inhibitors for the treatment of influenza infections in immunocompromised patients.

References