Frequency of Amantadine-Resistant Influenza A Viruses during Two Seasons Featuring Cocirculation of H1N1 and H3N2

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In two influenza seasons during which H1N1 and H3N2 cocirculated, resistance was more frequent in H3N2 strains than in H1N1 strains after amantadine treatment. Predominant amino acid substitutions in M2 protein occurred at position 31 (serine to asparagine) in H3N2 strains and at position 27 (valine to alanine) in H1N1 strains.

Amantadine is used for prevention and treatment of influenza A infections (2). The drug inhibits virus replication during the early stage of infection by blocking the ion channel formed by the M2 protein. One of the four amino acid substitutions at positions 26, 27, 30, and 31 within the transmembrane domain of the M2 protein has been implicated in loss of sensitivity to M2 inhibitors (6). In Japan, the usage of amantadine increased rapidly after its approval in 1998, and emergence of resistant viruses is now a major concern (12). Viral resistance to amantadine and its analogue, rimantadine, emerges quickly in vivo and in vitro, and we have found resistant viruses being circulated in nursing homes where amantadine was used not only for influenza virus but also for Parkinson’s disease (9, 11). In these reports, we demonstrated that 80 to 90% of elderly patients who shed resistant strains had no known prophylactic or therapeutic amantadine treatment during the study periods. Thus, the resistant strains appeared to be virulent, genetically stable, and capable of competing with wild, drug-sensitive strains of virus causing infection in humans. Using a 50% tissue culture infective dose (TCID₅₀)/0.2-ml drug-sensitive strains of virus causing infection in humans.

Virulence, School of Medicine. Supernatants of nasopharyngeal swabs were inoculated into MDCK cells for influenza virus isolation (10). Subtypes were determined by hemagglutination-inhibition tests with type-specific antisera (11), and amantadine-resistant viruses were assessed by TCID₅₀/0.2-ml titration with the strains isolated (9). Amino acid substitutions were detected simultaneously by the PCR-RFLP method (11). Resistant strains were confirmed by partial nucleotide sequencing of the transmembrane domain of the M2 protein (9, 11).

The overall frequencies of amantadine-resistant strains were 29.6% (24 of 81) during the 1999-2000 influenza season and 23.3% (7 of 30) during the 2000-2001 influenza season (Table 1). H1N1 and H3N2 influenza A viruses cocirculated during both seasons, but H3N2 strains predominated during the first season, and H1N1 predominated during the second. Resistant strains were detected more frequently from H3N2 strains (22 out of 66 [33.3%]) than from H1N1 strains (9 out of 45 [20.0%]) during both seasons, but the difference was not statistically significant. A total of 6 of 9 (66.7%) resistant H1N1 strains had an amino acid substitution at position 27 (valine to alanine [Val-27→Ala]), while 17 of 22 (77.3%) resistant H3N2 strains demonstrated a serine-to-asparagine change at position 31 (Ser-31→Asn) (Table 1). There were significant differences in the amino acid substitutions between subtypes and within H3N2 strains (27 versus 31) (Table 1).

Up to approximately one-third of patients shed resistant strains when amantadine or rimantadine was used for therapy (3, 5, 9, 11). We found patients with H3N2 strain infections shed more resistant viruses than their counterparts with H1N1. Previous studies were carried out mostly during H3N2 epidemics (3, 5, 7–9, 11), but the limited results concerning H1N1 and

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TABLE 1. Subtype-specific frequency of amantadine-resistant H1N1 and H3N2 strains from posttreatment samples during the 1999-2000 and 2000-2001 influenza seasons in Niigata City, Japan

<table>
<thead>
<tr>
<th></th>
<th>H1N1 strains</th>
<th></th>
<th>H3N2 strains</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of strains with amino acid substitution at:</td>
<td>No. of total resistant strains/isolates (%)</td>
<td>No. of strains with amino acid substitution at:</td>
<td>No. of total resistant strains/isolates (%)</td>
<td>Total by season (%)</td>
</tr>
<tr>
<td>Season</td>
<td>26</td>
<td>27</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>1999-2000</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2000-2001</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total by subtype (%)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

a Each number indicates the number of cases of amantadine-resistant influenza virus A strains with the respective amino acid substitutions in the transmembrane domain of the M2 protein.
b The proportion of amino acid substitutions at position 27 was significantly higher in H1N1 strains than in H3N2 strains (Yates corrected \( \chi^2 \) test, \( P < 0.01 \)).
c The proportion of amino acid substitutions at position 31 was significantly higher in H3N2 strains than in H1N1 strains (Yates corrected \( \chi^2 \) test, \( P < 0.05 \)).
d The proportion of amino acid substitutions at position 31 was significantly higher than at positions 27 and 30 within H3N2 strains (Yates corrected \( \chi^2 \) test, \( P < 0.001 \), respectively).

H3N2 cocirculation periods similarly pointed to a low level of emergence of amantadine-resistant H1N1 strains (1, 4). To date, no mechanism for the low level of emergence of amantadine-resistant H1N1 strains during cocirculation with the H3N2 strains has been suggested. All naturally occurring influenza A viruses are perhaps mixtures of resistant and sensitive strains, which are estimated to occur at a rate of 1 in 10,000, later becoming selected within 2 to 3 days of the start of amantadine therapy. In Japan, a large quantity of the drug has been used for treatment of influenza A virus infections since 1998. During the two influenza seasons investigated since 1999, in Niigata, Japan, H3N2 predominated in the first and H1N1 cocirculated in the second. Therefore, we speculate that the under the epidemiological conditions described above, amantadine-resistant H3N2 strains may appear and survive more frequently, although this requires confirmation.

We also found dominant amino acid substitution residues differ significantly with the subtype: namely, Val-27 → Ala in H1N1 strains and Ser-31 → Asn in H3N2 strains. A thorough search of the literature and our previous reports indicated that 70 to 80% of substitutions in amantadine-resistant viruses occur at position 31, and around 10% each occur at positions 27 and 30 in H3N2 strains in vitro (9) and in clinical samples (9, 11). However, earlier reports did not examine those subtype differences. For PCR-RFLP analysis, we performed nested PCR with three pairs of primers corresponding to amino acid substitutions at positions 27, 30, and 31. For quick surveys, it might be of advantage to select specific primers for substitution at position 27 for H1N1 strains and at position 31 for H3N2.

With regard to planning for treatment of pandemics caused by new virulent influenza virus strains, a novel class of antiviral agent, neuraminidase inhibitors, is promising candidate for treatment and prophylaxis, but amantadine is still an important option regarding cost and chemical stability. Thus, the monitoring of amantadine resistance should be maintained together with investigations of pathogenicity, transmissibility, and frequency in vivo.

This study suggests that viral resistance to amantadine was more frequent in H3N2 strains than in H1N1 strains after amantadine treatment. The predominant amino acid substitutions in M2 protein occurred at position 31 (serine to asparagine) in H3N2 strains and at position 27 (valine to alanine) in H1N1 strains. This study underlines the need for further investigations to elucidate the reasons for subtype differences in generation of amantadine-resistant strains.

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REFERENCES


