

Emergence of Influenza B Viruses With Reduced Sensitivity to Neuraminidase Inhibitors

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CLINICAL USE OF ANY ANTIVIRAL drug can lead to the development of drug-resistant viruses.^{1,2} Two neuraminidase inhibitors, zanamivir and oseltamivir, are effective against influenza and are used extensively for treatment of this infection, especially in Japan.^{3,4} We and others have documented the emergence of oseltamivir-resistant type A viruses, including H5N1 subtypes,^{3,5-7} but similar information on influenza B viruses is limited. Although influenza B viruses usually cause smaller epidemics than type A viruses, they are nonetheless associated with annual outbreaks of illness and excess mortality rates worldwide.⁸

Of the 2 type B viruses with reduced sensitivity that have been reported, 1 carried an Arg152Lys mutation (amino acid numbering system adapted for an N2 neuraminidase⁹; N2 numbering is

Context Very little is known about the frequency of generation and transmissibility of influenza B viruses with reduced sensitivity to neuraminidase inhibitors. Furthermore, transmission of resistant virus, whether influenza A or B, has not been recognized to date.

Objective To assess the prevalence and transmissibility of influenza B viruses with reduced sensitivity to neuraminidase inhibitors.

Design, Setting, and Patients Investigation of the neuraminidase inhibitor sensitivity of influenza B isolates from 74 children before and after oseltamivir therapy and from 348 untreated patients with influenza (including 66 adults) seen at 4 community hospitals in Japan during the 2004-2005 influenza season. Four hundred twenty-two viruses from untreated patients and 74 samples from patients after oseltamivir therapy were analyzed.

Main Outcome Measure Sialidase inhibition assay was used to test the drug sensitivities of influenza B viruses. The neuraminidase and hemagglutinin genes of viruses showing reduced sensitivity to neuraminidase inhibitors were sequenced to identify mutations that have the potential to confer reduced sensitivity to these drugs.

Results In 1 (1.4%) of the 74 children who had received oseltamivir, we identified a variant with reduced drug sensitivity possessing a Gly402Ser neuraminidase substitution. We also identified variants with reduced sensitivity carrying an Asp198Asn, Ile222Thr, or Ser250Gly mutation in 7 (1.7%) of the 422 viruses from untreated patients. Review of the clinical and viral genetic information available on these 7 patients indicated that 4 were likely infected in a community setting, while the remaining 3 were probably infected through contact with siblings shedding the mutant viruses.

Conclusions In this population, influenza B viruses with reduced sensitivity to neuraminidase inhibitors do not arise as frequently as resistant influenza A viruses. However, they appear to be transmitted within communities and families, requiring continued close monitoring.

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used throughout this article) in its neuraminidase and was isolated from an immunocompromised child treated with

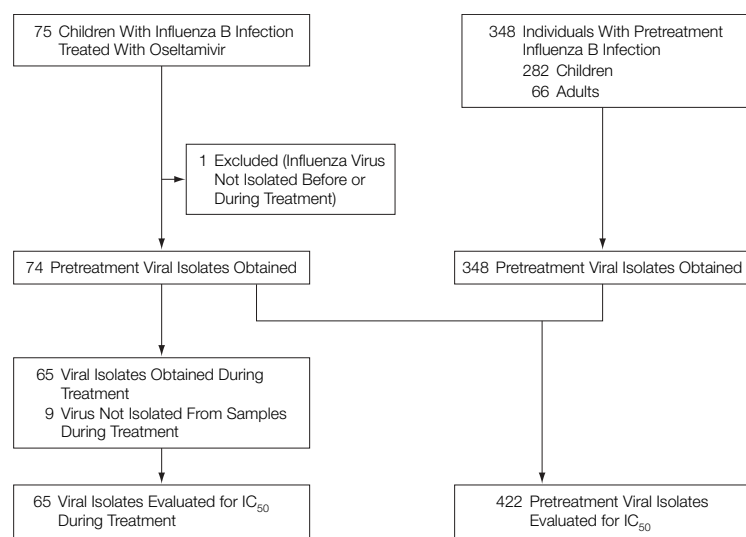
zanamivir.¹⁰ The other virus had an Asp198Asn neuraminidase mutation and was isolated from an immunocompro-

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For editorial comment see p 1492.

Figure 1. Flowchart of Participants

IC₅₀ indicates concentration of neuraminidase inhibitor required to inhibit 50% of the sialidase activity of influenza B viruses.

mised child treated with oseltamivir.¹¹ The known neuraminidase substitutions identified in drug-resistant viruses from humans tend to be type- or subtype-specific: Glu119Val, Arg292Lys, and Asn294Ser in the neuraminidase of the N2 subtype; His274Tyr in that of the N1 subtype (including not only H1N1 viruses but also H5N1 viruses)^{6,7}; and Arg152Lys and Asp198Asn in that of the type B virus.^{10,11} All of these substitutions have been identified at catalytic or framework residues in the sialidase active sites of the neuraminidase protein,⁹ which are relatively conserved in all type A and type B neuraminidase molecules and are the targets of neuraminidase inhibitors.

The results of cell culture experiments in which multiple passages were required for the generation of viruses resistant to neuraminidase inhibitors¹² have suggested that resistance to these agents arises infrequently. It is thus reasonable that a low frequency of oseltamivir resistance—5.5% for children aged 1 to 12 years infected with type A viruses and 0% in children infected with type B viruses—was observed in a clinical trial.¹³ More recent studies, however, have demonstrated a higher-than-expected rate of drug-resistant influ-

enza A virus generation in oseltamivir-treated children: 18% of children with H3N2 virus infection⁵ and 16% of those with H1N1 virus infection³ harbored resistant variants with neuraminidase mutations after drug treatment.

The biological fitness of viruses resistant to neuraminidase inhibitors differs depending on the type of mutations in the neuraminidase. In mouse or ferret studies, the infectivity and transmissibility of clinical isolates of human influenza A viruses carrying the Arg292Lys or the His274Tyr mutation in their neuraminidases were compromised¹⁴⁻¹⁷; a similar result was reported for a mutant type B virus with the Arg152Lys mutation in ferrets.¹⁰ By contrast, a resistant virus with the Glu119Val mutation infected ferrets and was transmitted among these animals as efficiently as the wild-type virus.¹⁷ Also, influenza B virus carrying the Asp198Asn substitution grows as well as the wild-type virus in this animal model.¹⁸ Nonetheless, the pathogenicity and transmissibility of neuraminidase inhibitor-resistant viruses remain unanswered questions with significance for predicting pandemic strains.

In Japan, the neuraminidase inhibitors zanamivir and oseltamivir were ap-

proved for clinical use in 2000 and 2001, respectively, and are now used more extensively there than anywhere else in the world.^{3,4} In the winter of 2004-2005, an influenza B virus caused a widespread epidemic in Japan, creating opportunities to assess in a natural setting the prevalence and transmissibility of influenza B viruses with reduced sensitivity to neuraminidase inhibitors. The results reported here suggest a low but appreciable rate of emergence of type B viruses with reduced sensitivity to neuraminidase inhibitors and their person-to-person transmission, both in the community and within single families.

METHODS

Study Population and Settings

Pharyngeal or nasal swabs for influenza B virus analysis were obtained from patients who visited the pediatric services at 4 community hospitals in Japan during the 2004-2005 influenza season. To identify the frequency of developing neuraminidase inhibitor-resistant influenza B viruses after oseltamivir therapy, we analyzed paired specimens from each patient, one taken at the initial hospital visit (pretreatment samples) and the other during treatment with oseltamivir (posttreatment samples). Patients diagnosed with influenza B virus infection by a rapid diagnostic kit who received oseltamivir therapy, and from whom we could obtain at least 2 sequential samples for virus isolation, were enrolled in the first series of studies (FIGURE 1). In the second series, we attempted to assess in a community setting the prevalence of influenza B viruses with reduced sensitivity to neuraminidase inhibitors. To this end, we obtained samples before oseltamivir treatment from patients who visited the 4 community hospitals. The influenza B viruses isolated from these samples and the viruses from the pretreatment samples from the first series of studies were combined and analyzed (Figure 1). Because these studies included patients who visited community hospitals, several family members sought consultation at the same facility.

Oral informed consent was obtained from the parents of all patients. This study was conducted with the approval of the ethics committees of 3 of the 4 hospitals; in the case of the single hospital in which an ethics committee did not exist, the activities of the study were undertaken under the auspices of the informed consent.

Clinical Specimens and Viruses

Pharyngeal or nasal swabs for influenza B virus isolation were obtained by attending physicians after informed consent was obtained. The viruses isolated were stored at -80°C until used. The viral isolates were used as mixed populations without plaque purification. Madin-Darby canine kidney cells overexpressing the β -galactoside $\alpha 2,6$ -sialyltransferase I (ST6Gal I) gene¹⁹ were used for viral isolation and plaque assay. These cells support the growth of clinical isolates of human influenza viruses better than unmanipulated Madin-Darby canine kidney cells.¹⁹ To assess the sensitivity of the influenza B viruses to neuraminidase inhibitors, the concentration of neuraminidase inhibitor required to inhibit 50% of the sialidase activity of the viruses (IC_{50}) was determined with pretreatment and posttreatment influenza B isolates using a sialidase inhibition assay.^{19,20} The IC_{50} values demonstrated in this study were assessed for viruses present in culture supernatant fluids, without plaque purification of the isolates. For strains demonstrating reduced susceptibility to the inhibitors, we sequenced their neuraminidase and hemagglutinin genes.

Sialidase Sensitivity to Neuraminidase Inhibitors

Sialidase sensitivities of influenza B viruses to neuraminidase inhibitors were evaluated with a sialidase inhibition assay as described previously.^{19,20} Briefly, 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA; Sigma, St Louis, Mo) at a final concentration of 0.1 mmol/L was used as a substrate. Ten microliters of the virus dilution (predetermined to contain si-

alidase activity in the range of 800-1200 fluorescence units in this assay) and 10 μL of the neuraminidase inhibitor (0.01 nmol/L to 10 $\mu\text{mol/L}$) in calcium-MES buffer (33 mmol/L 2-[N-morpholino]ethanesulfonic acid, 4 mmol/L CaCl_2 , pH 6.0) were mixed and incubated at 37°C for 30 minutes, followed by the addition of 30 μL of the substrate. The mixtures were further incubated at 37°C for 60 minutes, and the reaction was stopped by adding 150 μL of 0.1 mol/L sodium hydroxide in 80% ethanol (pH 10.0). We quantified fluorescence at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The IC_{50} value was determined by extrapolation of the relation between the concentration of inhibitor and the proportion of fluorescence inhibition. Results are reported as the mean of duplicate IC_{50} values. Oseltamivir carboxylate (GS4071; Roche Products, Basel, Switzerland), the active metabolite of the ethyl ester prodrug oseltamivir phosphate, and zanamivir (Relenza; GlaxoSmithKline, Brentford, UK) were used as neuraminidase inhibitors.

Sequence Analyses of the Neuraminidase and Hemagglutinin Genes

Viral RNA was extracted from virus in cell-culture supernatant fluid with an RNA extraction kit (ISOGEN-LS; Nippon Gene, Tokyo, Japan), without prior plaque purification of the virus. Reverse transcription was performed with reverse transcriptase (SUPERScript III; Invitrogen, Carlsbad, Calif) and a primer complementary to the 3' end of the viral RNA (5'-AGCAGAAAG-CAGAGCA-3'). The cDNA products were then used to amplify the viral neuraminidase and hemagglutinin genes by a standard polymerase chain reaction method (Pfu Ultra DNA Polymerase; Stratagene, La Jolla, Calif) (primer sequences and amplification conditions available from the authors on request). We cloned these products into the pCRBlunt II-TOPO vector (Invitrogen) and transformed them into TOP10 chemically competent cells

(Invitrogen). Transformed cells were grown on Luria broth agar containing 50 mg/L of kanamycin, after which the kanamycin-resistant colonies were selected and incubated in Luria broth at 37°C overnight in a shaking incubator. Plasmid DNA was extracted with the MagExtractor-plasmid system (TOYOBO, Osaka, Japan). We determined complete sequences of the neuraminidase and hemagglutinin genes by cycle sequencing with dye-terminator chemistry (Big Dye; Applied Biosystems, Foster City, Calif) on the Applied Biosystems 3100 or 3130XL auto sequencer using M13F-20, neuraminidase-specific, or hemagglutinin-specific primers. For each sample, 5 to 8 cDNA clones of the neuraminidase and hemagglutinin genes were analyzed.

RESULTS

Study Population

A total of 75 pairs of pretreatment and posttreatment samples were obtained from pediatric patients. One pair was excluded because influenza virus was not isolated from either the pretreatment or posttreatment sample. Thus, 74 patients aged 0 to 15 years (median, 3 years) with influenza B virus infection were enrolled in the study (Figure 1). All were treated with oseltamivir for 5 days. Eighteen children received 2 mg/kg of body weight twice daily, while the remaining 56 children received weight-based unit doses²¹ (body weight ≤ 15 kg, 30 mg twice daily; >15 -23 kg, 45 mg twice daily; >23 -40 kg, 60 mg twice daily; and >40 kg, 75 mg twice daily).

In the second series of experiments, we analyzed a total of 442 influenza B viruses isolated from patients prior to treatment (348 patients plus the above-mentioned 74 patients) during the 2004-2005 influenza season (Figure 1). Of the 422 patients, 356 were children aged 0 to 15 years (median, 5 years); the remaining 66 were adults 16 years or older (range, 17 to 61 years; median, 34 years).

Emergence of Influenza B Viruses With Reduced Sensitivity to Neuraminidase Inhibitors After Oseltamivir Treatment

Viruses were recovered from all pre-treatment samples and from 65 post-treatment samples collected from the 74 children who had received a full course of oseltamivir. In 1 child (1.4%), the IC₅₀ value of the posttreatment isolate tested against zanamivir and oseltamivir increased by 7.1-fold and 3.9-fold, respectively, compared with results for the virus isolated before treatment (TABLE, patient 1). This child was an immunocompetent 7-year-old boy who had received oseltamivir immediately after diagnosis. The virus with reduced sensitivity to the neuraminidase inhibitors was isolated from a pharyngeal swab collected on day 3 after initiation of oseltamivir therapy. To understand the molecular basis of the observed reduced sensitivity to the drugs, we molecularly cloned the neuraminidase gene from the virus exhibiting this property. The sequence analysis revealed an amino acid substitution, Gly402Ser, in 7 of the 8 cDNA clones of the neuraminidase gene. No other difference was observed in the amino acid sequence of the neuraminidase and hemagglutinin proteins between the

wild-type parent and the posttreatment mutant virus. The neuraminidase mutation Gly402Ser was located near the sialidase enzymatic center.

Influenza B Viruses With Reduced Susceptibility to Neuraminidase Inhibitors Detected in Patients Prior to Treatment

The median (interquartile range) IC₅₀ values for influenza B viruses isolated from 422 untreated patients during the 2004-2005 influenza season and tested against both oseltamivir and zanamivir with the sialidase inhibition assay were 70.5 (55.8-85.1) nmol/L and 10.1 (7.0-15.8) nmol/L, respectively (FIGURE 2). Considering the level of increase in the IC₅₀ value of the virus from the posttreatment sample as compared with that of the original virus obtained before oseltamivir therapy from patient 1, we regarded viruses whose IC₅₀ values were higher than 1.5 times interquartile range above the third quartile as drug-resistant (Figure 2). Using this criterion, 7 (1.7%) of the 422 influenza B viruses isolated from untreated patients (Table, patients 2-8) were found to have reduced sensitivity to zanamivir, oseltamivir, or both. Each of the 7 isolates with reduced sensitivity contained amino acid substitu-

tions in the neuraminidase at the sialidase active center, by comparison with the consensus type B neuraminidase sequence: 3 had Asp198Asn mutations, 3 had Ile222Thr mutations, and 1 had a Ser250Gly mutation (Table). None of these patients had an underlying disease and none had received immunosuppressive drugs.

An 8-year-old boy (patient 2) was diagnosed with influenza B virus infection 6 days before the onset of influenza B infection in his 1-year-old sister (patient 3). The IC₅₀ values for the pre-treatment isolate from patient 2 (47.4 nmol/L for zanamivir and 237.3 nmol/L for oseltamivir) indicated reduced sensitivity of the isolate to these compounds. We identified a neuraminidase mutation at position 198 (Asp198Asn) in all of the 8 cDNA clones of the neuraminidase gene of this isolate. The virus isolated from patient 3 also showed reduced sensitivity to zanamivir and oseltamivir (Table). Sequence analyses of the neuraminidase and hemagglutinin genes were identical between viruses isolated from patients 2 and 3, including the presence of an Asp198Asn mutation in the neuraminidase protein (in all of the 8 cDNA clones of the neuraminidase gene of the isolate from patient 3). Thus, it

Table. Influenza B Isolates With Reduced Sensitivity to Neuraminidase Inhibitors Before or After Antiviral Treatment

Patient No.	Age, y	Sex	Sample	Date of Isolation	IC ₅₀ , nmol/L		Mutations Found in Neuraminidase*
					Zanamivir	Oseltamivir	
1†	7	Male	Pretreatment	2/19/05	6.6	72.3	None
			Posttreatment	2/21/05	46.9	280.6	Gly402Ser
2‡	8	Male	Pretreatment	2/22/05	47.4	237.3	Asp198Asn
			Posttreatment	2/28/05	42.2	228.2	Asp198Asn
3§	1	Female	Pretreatment	2/28/05	48.9	255.3	Asp198Asn
			Posttreatment	3/5/05	51.3	239.7	Asp198Asn
4	6	Female	Pretreatment	2/9/05	61.7	204.2	Asp198Asn
5‡	6	Male	Pretreatment	2/9/05	23.3	443.0	Ile222Thr
6¶	3	Female	Pretreatment	2/16/05	29.5	479.9	Ile222Thr
7‡	5	Male	Pretreatment	2/7/05	22.6	513.8	Ile222Thr
8‡	22	Female	Pretreatment	1/22/05	191.3	48.6	Ser250Gly

Abbreviation: IC₅₀, concentration of neuraminidase inhibitor required to inhibit 50% of the sialidase activity of influenza B viruses.

*Amino acid differences were identified by comparison with the consensus sequence of currently circulating type B viruses. Amino acid numbering was adapted to that of the N2 neuraminidase. Positions 198, 222, 250, and 402 in N2 neuraminidase correspond to positions 197, 221, 249, and 407, respectively, in type B neuraminidase.

†Patient 1 received oseltamivir from February 19 to February 23, 2005.

‡Onset of symptoms was not preceded by infection of other family members.

§Sister of patient 2.

||Four-year-old sister of this patient received oseltamivir for wild-type influenza B virus infection from January 31 (date of isolation) to February 4, 2005, but virus isolation after oseltamivir therapy was not performed.

¶Sister of patient 5.

may be possible that patient 2 was infected with an influenza B virus having reduced sensitivity to neuraminidase inhibitors and then transmitted the virus to his sister, patient 3.

Another influenza B virus possessing the Asp198Asn mutation in the neuraminidase was isolated from patient 4 (aged 6 years, female) on February 9, 2005, before oseltamivir treatment (Table). This neuraminidase mutation was observed in all of the 7 cDNA clones of this isolate. Her 4-year-old sister, from whom a wild-type influenza B virus had been isolated on January 31, 2005, had received oseltamivir from January 31 to February 4 (Table). The sequences of both the neuraminidase and hemagglutinin genes from the 2 patients were identical, with the exception of a neuraminidase substitution at amino acid position 198. Thus, it is possible that a drug-resistant virus might have arisen in the 4-year-old sister during oseltamivir therapy and been transmitted to patient 4. However, because we were unable to obtain samples after oseltamivir therapy from the 4-year-old

sister, we cannot prove that this was indeed the case.

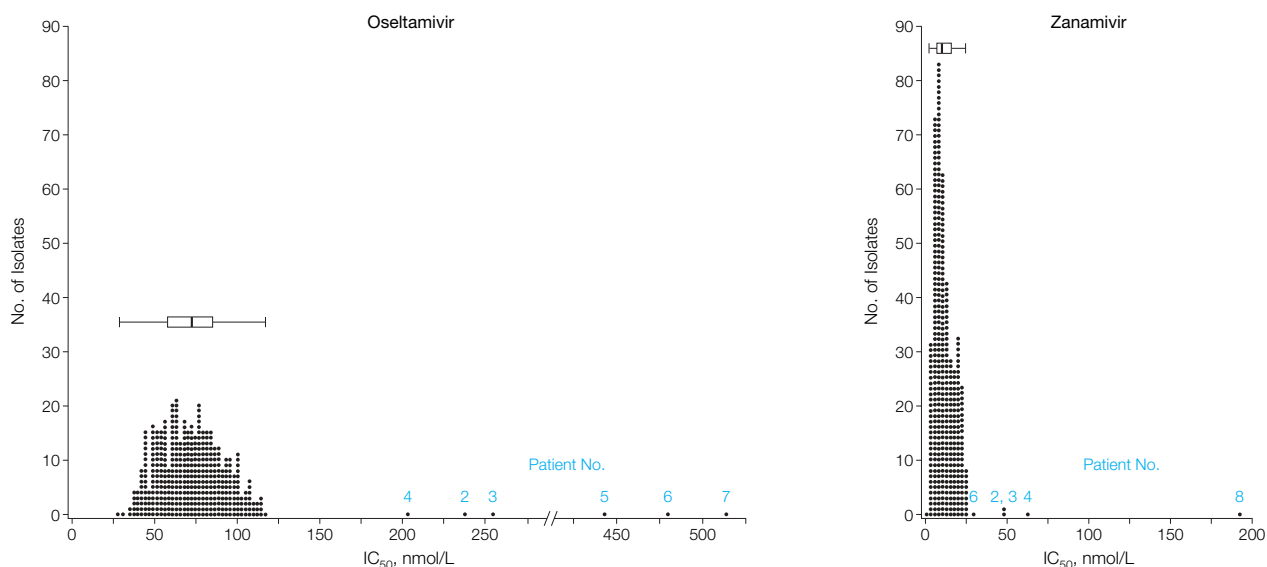
The IC_{50} values for the Asp198Asn mutants ranged from 42 to 62 nmol/L (zanamivir) and from 204 to 255 nmol/L (oseltamivir), indicating that the mutation was associated with approximately 3- to 4-fold and 4- to 6-fold reductions in drug sensitivity, respectively, compared with the corresponding median IC_{50} values for the entire group of type B viruses. The variant with reduced sensitivity to oseltamivir and with the Asp198Asn mutation was recently identified by Gubareva¹¹ and by Mishin et al¹⁸ in a posttreatment sample from an immunocompromised child with influenza B virus, further supporting the notion that this mutation was introduced during oseltamivir therapy and that it reduced sensitivity to the neuraminidase inhibitors.

Several type B viruses carrying other neuraminidase mutations with reduced sensitivity were also identified in other patients. Viruses carrying an Ile222Thr mutation were isolated from pretreatment samples of 3 patients: patients 5 and

6 (siblings) and patient 7 (Table). The nucleotide sequences of the neuraminidase and hemagglutinin genes of isolates from these patients were identical, and the neuraminidase Ile222Thr mutation was observed in all of the cDNA clones of each viral NA gene. The IC_{50} values for viruses carrying the Ile222Thr mutation ranged from 443 to 514 nmol/L (oseltamivir), representing a 6- to 7-fold reduction in sensitivity compared with the median IC_{50} values for type B viruses (Table). This mutation appeared to lack strong effect on viral sensitivity to zanamivir. An influenza B virus with reduced sensitivity to the neuraminidase inhibitors was also isolated from patient 8, a 22-year-old woman (Table). The isolate from patient 8 possessed a Ser250Gly mutation in all of the 7 cDNA clones of the neuraminidase gene. The Ser250Gly mutation conferred an approximately 19-fold resistance to zanamivir (when compared with the median type B virus IC_{50} value) but did not reduce sensitivity to oseltamivir.

None of the family members of patients 2, 5, 7, and 8 were affected by in-

Figure 2. Distribution of IC_{50} Values for Influenza B Viruses Isolated From 422 Patients Prior to Treatment, Tested Against Oseltamivir Carboxylate and Zanamivir



IC_{50} indicates concentration of neuraminidase inhibitor required to inhibit 50% of the sialidase activity of influenza B viruses. The IC_{50} values were determined by the sialidase inhibition assay.^{19,20} Boxes indicate interquartile ranges (IQRs); vertical lines, median values; and bars to the left and right of boxes, 1.5 times IQR. The median (IQR) IC_{50} values of the 422 type B viruses from patients before treatment were 70.5 (55.8-85.1) nmol/L for oseltamivir and 10.1 (7.0-15.8) nmol/L for zanamivir. Patient numbers in blue indicate those untreated patients with influenza B viral isolates found to have reduced sensitivity to oseltamivir (left) and/or zanamivir (right).

fluenza B virus before onset of their symptoms, suggesting that they were possibly infected with mutants with reduced drug sensitivity circulating in the community. These results suggest that influenza B viruses with reduced sensitivity to neuraminidase inhibitors might possibly be transmitted from person to person, not only within single families but also among members of the same community.

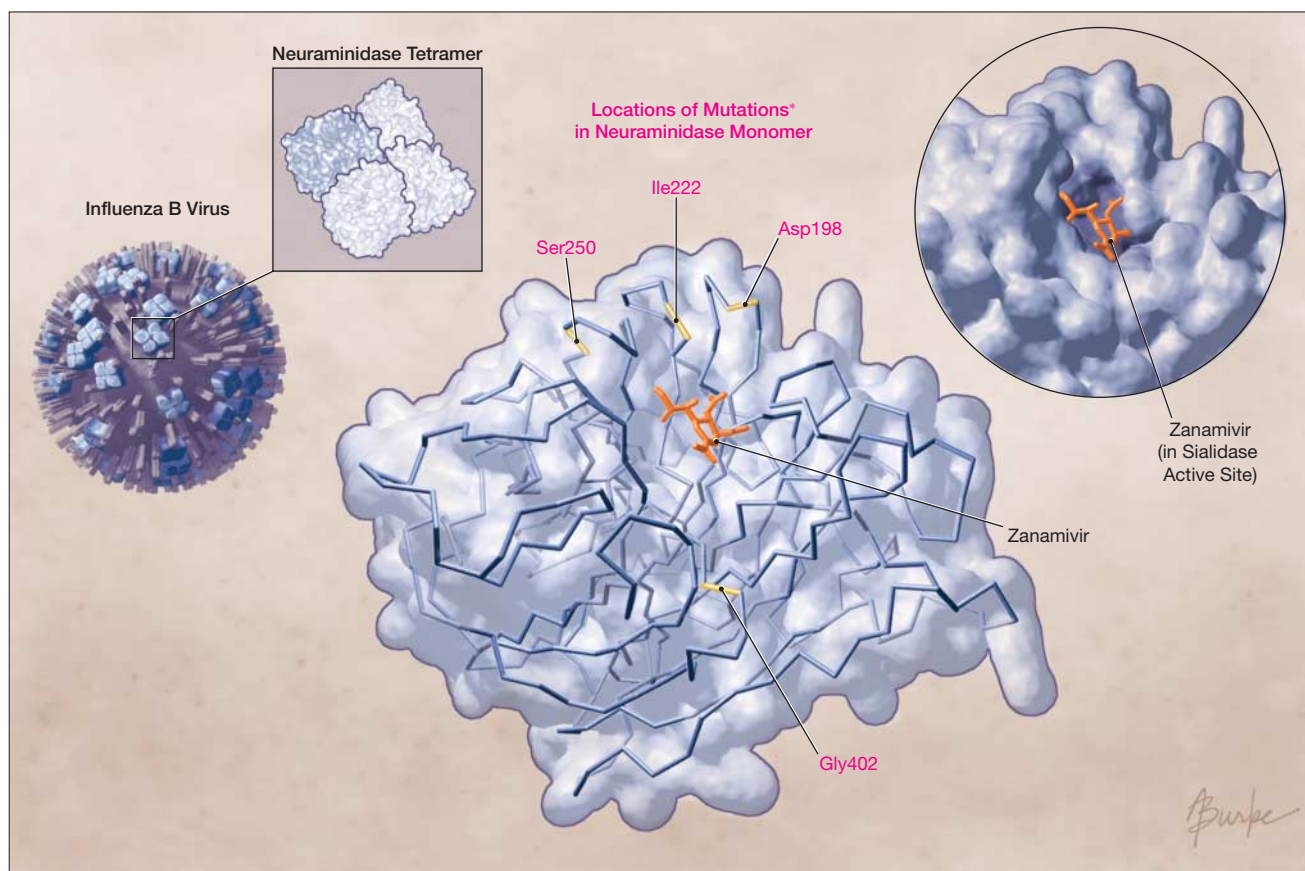
Finally, we did not observe any appreciable differences in the clinical course of viral infection between patients infected with wild-type viruses or those with reduced sensitivity to neuraminidase inhibitors. Mean durations of fever after antiviral therapy were 2.4, 2.6,

and 2.0 days in patients infected with wild-type viruses ($n=32$), those infected with reduced sensitivity to neuraminidase inhibitors (patient 2 [3 days], patient 3 [5.5 days], patient 7 [1 day], patient 8 [1 day]), and the patient with the variant that developed during therapy (patient 1), respectively. Similarly, we did not find an appreciable difference in the extent of virus shedding (duration and titer) between patients infected with a drug-resistant virus and those infected with a drug-sensitive virus. However, the number of patients infected with viruses with reduced drug sensitivity is too small to assess the statistical significance of the effect of drug resistance on virus shedding.

COMMENT

We demonstrated that influenza B viruses with reduced sensitivity to neuraminidase inhibitors can emerge during routine therapy and that such mutants appear to be transmitted from person to person, not only within the same family but possibly through community contacts as well. The rate of generation of influenza B viruses with reduced drug sensitivity during oseltamivir treatment in this study, 1.4%, is lower than that among influenza A viruses (5.5%-18%).^{3,5,13} This discrepancy could reflect the higher doses of oseltamivir used in our study (76% of the patients received weight-based unit doses of the drug, in con-

Figure 3. Locations of Mutations on the 3-Dimensional Structure of Neuraminidase



Three-dimensional structure of the complex between influenza virus B/Beijing/1/87 neuraminidase and zanamivir (Molecular Modeling DataBase Identifier: 10147; Protein DataBank Identifier: 1A4G). The locations of the neuraminidase mutations* identified in the present study (aspartic acid 198 [Asp198], isoleucine 222 [Ile222], serine 250 [Ser250], and glycine 402 [Gly402]) that are associated with decreased drug sensitivity are marked in yellow. These mutations are located at or near the sialidase active site, where neuraminidase inhibitors bind. (See online interactive neuraminidase model at <http://jama.com/cgi/content/full/297/13/1435/DC1>.)

*Amino acids are numbered according to the N2 NA numbering system. Corresponding influenza virus B/Beijing/1/87 positions are Asp196, Ile220, Ser248, and Gly406; for currently circulating type B viruses, Asp197, Ile221, Ser249, and Gly407, respectively.

trast to the twice-daily 2 mg/kg dose uniformly administered in previous Japanese studies^{3,5}).

Four mutations in the type B neuraminidase reduced sensitivity to neuraminidase inhibitors: Asp198Asn, Ile222Thr, Ser250Gly, and Gly402Ser substitutions. Residues 198, 222, and 250 are located in the framework of the neuraminidase active site, which interacts with the catalytic residues by hydrogen bonds or salt bridges (FIGURE 3).^{9,22} The framework residues Asp198 and Ser 250 (the corresponding residue in the type A neuraminidase is Ala) interact with the catalytic residues Arg152 and Arg224, respectively, and Ile222 forms a hydrophobic pocket into which the substrate fits.²² The novel substitution detected in the neuraminidase of a virus recovered from an oseltamivir-treated patient in this study occurred at residue 402. Although Gly402 is not a catalytic or framework residue, it is located near the sialidase enzymatic center (Figure 3). Therefore, Gly402Ser substitution may alter the interaction of the enzymatic center and the neuraminidase inhibitors, resulting in reduced drug sensitivity. Further analysis is needed to understand the interaction of this residue with sialic acid.

The framework mutations we identified appear to reduce oseltamivir sensitivity at a moderate level as compared with the catalytic Arg292Lys mutation. When tested against oseltamivir, the IC₅₀ values for H3N2 viruses with the framework mutation Glu119Val or Asn294Ser were 239 nmol/L or 106 nmol/L,⁵ respectively, while that for an H5N1 strain with the framework mutation His274Tyr was 763 nmol/L.⁶ On the other hand, the catalytic Arg292Lys mutation in N2 viruses conferred a high level of resistance to oseltamivir (IC₅₀ >10 000 nmol/L).⁵ Viruses with framework mutations might have the ability to be transmitted among experimental animals, as has been shown with type A variants with a framework mutation at position 119 or 274.¹⁷ These results suggest that influenza viruses with a framework mutation in the neur-

aminidase might be of clinical concern, even though their IC₅₀ values are lower than those of viruses with mutations in the catalytic domain. Thus, recent reports of oseltamivir resistance in H5N1 influenza A viruses harboring the framework His274Tyr mutation^{6,7} warrant particular attention and careful monitoring for the spreading of resistant variants.

The question remains as to whether the variants isolated from untreated patients demonstrate person-to-person transmissibility in a community or the spontaneous emergence of mutants with reduced drug sensitivity. We favor the first possibility because the global Neuraminidase Inhibitor Susceptibility Network did not identify influenza viruses with resistance to neuraminidase inhibitors before these drugs were introduced into clinical use.^{23,24} However, in the first 3 influenza seasons (1999-2002) following the introduction of neuraminidase inhibitors to the market, the network detected a small number (8 [0.35%] of 2287 isolates) of influenza viruses, isolated from untreated patients, with decreased susceptibility to neuraminidase inhibitors.²⁵ Of those, 2 possessed neuraminidase mutations previously identified in neuraminidase inhibitor-resistant viruses. Moreover, in the 2003-2004 influenza season, the network identified 3 H3N2 viruses in 1180 samples collected in Japan that contained neuraminidase mutations conferring resistance to neuraminidase inhibitors, although it was not possible to determine with certainty whether these patients had been exposed to neuraminidase inhibitors or to neuraminidase inhibitor-treated individuals.²⁶ Our findings are consistent with these surveillance data, which imply a possible transmission of neuraminidase inhibitor-resistant viruses from person to person.

When healthy children were given oseltamivir at 2 mg/kg of body weight, the mean peak plasma concentration of oseltamivir carboxylate, the active metabolite of the drug, was 630 nmol/L among children aged 3 to 5 years and 426 nmol/L among children aged 1 to 2 years.²⁷ This indicates that the IC₅₀ val-

ues for influenza B viruses tested against oseltamivir in our study were close to the plasma drug concentration, suggesting that this drug may not be as effective against influenza B virus as against influenza A virus. By contrast, the topical concentration of zanamivir in the human respiratory tract is estimated to be more than 10 000 nmol/L when healthy adults inhale 10 mg zanamivir,²⁸ well above the influenza B virus IC₅₀ values.

In Japan, prescriptions for oseltamivir were estimated to be 90 times more common than those for zanamivir during the 2004-2005 influenza season (official notice from the Ministry of Health, Labor and Welfare of Japan). It is therefore possible that the mutants with reduced drug sensitivity found in communities in this study had been generated by widespread use of oseltamivir. Continued surveillance for the emergence or spread of neuraminidase inhibitor-resistant influenza viruses is critically important.

Finally, the clinical course of influenza B virus infection in this study did not appear to be affected by the sensitivity of the virus to neuraminidase inhibitors, although larger numbers of cases will need to be studied to confirm this impression. Nonetheless, the symptoms of patients infected with viruses with reduced sensitivities to neuraminidase inhibitors were similar to those of patients infected with wild-type viruses, indicating that these mutant viruses, at least those carrying the framework mutation, do not lose virulence even though they have evolved to a status that is less sensitive to the drug. Further evaluation of the biological properties of neuraminidase inhibitor-resistant influenza viruses is needed to fully assess their pathogenicity in humans.

Author Contributions: Drs Hatakeyama and Kawaoka had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data: Hatakeyama, Ito, Yamazaki, Ichikawa, Kimura, Kiso, Shimizu, Kawakami, Mitamura. **Analysis and interpretation of data:** Hatakeyama, Koike, Kawaoka.

Drafting of the manuscript: Hatakeyama.

Critical revision of the manuscript for important intellectual content: Sugaya, Ito, Yamazaki, Ichikawa, Kimura, Kiso, Shimizu, Kawakami, Koike, Mitamura, Kawaoka.

Statistical analysis: Hatakeyama.

Obtained funding: Kawaoka.

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