

Frequency of Drug-resistant Viruses and Virus Shedding in Pediatric Influenza Patients Treated With Neuraminidase Inhibitors

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(See editorial commentary by Dolan on pages 438–439.)

Background. Although influenza virus resistance to the neuraminidase inhibitor zanamivir is reported less frequently than is resistance to the neuraminidase inhibitor oseltamivir in clinical settings, it is unknown whether this difference is due to the limited use of zanamivir or to an inherent property of the drug. We therefore compared the prevalence of drug-resistant viruses and virus shedding in seasonal influenza virus–infected children treated with either oseltamivir or zanamivir.

Methods. Clinical specimens (throat or nasal swab) were collected from a total of 144 pediatric influenza patients during the 2005–2006, 2006–2007, 2007–2008, and 2008–2009 influenza seasons. Neuraminidase inhibitor–resistant mutants were detected among the isolated viruses by sequencing the viral hemagglutinin and neuraminidase genes. Sensitivity of the viruses to neuraminidase inhibitors was tested by neuraminidase inhibition assay.

Results. In oseltamivir- or zanamivir-treated influenza patients who were statistically comparable in their age distribution, vaccination history, and type or subtype of virus isolates, the virus-shedding period in zanamivir-treated patients was significantly shorter than that in oseltamivir-treated patients. Furthermore, the frequency of zanamivir-resistant viruses was significantly lower than that of oseltamivir-resistant viruses.

Conclusion. In comparison with treatment with oseltamivir, treatment of pediatric patients with zanamivir resulted in the emergence of fewer drug-resistant influenza viruses and a shorter virus-shedding period. We conclude that zanamivir shows promise as a better therapy for pediatric influenza patients.

Seasonal influenza imposes substantial disease burden, particularly to high-risk populations—pediatric, geriatric,

and immunocompromised populations, among others—who bear a substantial proportion of the morbidity and mortality of the disease [1–8]. The development of zanamivir and oseltamivir, neuraminidase (NA) inhibitors of influenza viruses, provided improved drug therapies to treat influenza patients [9–11]. The efficacies of these 2 NA inhibitors were comparable [12–14]. However, oseltamivir, an oral drug, has been used more extensively than has zanamivir, an inhalant drug.

The emergence of drug-resistant influenza viruses is a major concern when antiviral drug therapies are used, because such viruses would nullify the drugs, as exemplified by the case of the recently emerged H1N1

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seasonal influenza A virus [15, 16]. The frequency of drug-resistant influenza viruses is lower in adults than children [17, 18], in whom limited immunity to these viruses leads to a protracted course of viral replication and thus to a higher probability of emergence of drug-resistant viruses [19–21]. The emergence of drug-resistant viruses in children affects not only individual patients but also public health.

Viruses resistant to NA inhibitors emerge less frequently than those resistant to the M2 ion channel inhibitors amantadine and rimantadine [22, 23], which have been used for the treatment of influenza patients for more than 2 decades [24, 25]. Although a single amino acid substitution at position 119, 136, 152, 274, 292, or 294 in the NA (N2 numbering system) confers resistance to viruses against oseltamivir and/or zanamivir [10, 19, 22, 26–28], viruses possessing these substitutions have been attenuated and thought not to cause epidemics [22, 29–31]. Studies, however, revealed a higher proportion of oseltamivir-resistant viruses among oseltamivir-treated pediatric patients than was originally expected [19], as well as person-to-person transmission of oseltamivir-resistant influenza B viruses [32]. Furthermore, oseltamivir-resistant human H1N1 viruses that efficiently transmit among humans emerged in Europe during the 2007–2008 influenza season [15], spread globally, and are currently circulating without selective pressure of antiviral compounds [16]. Recently, the effectiveness of oseltamivir was shown to be decreased among pediatric influenza patients infected with oseltamivir-resistant viruses [33]. Amino acid substitutions in the hemagglutinin (HA) are also known to decrease the sensitivity of viruses to NA inhibitors [29, 34].

Although during the 2005–2006 influenza season in Japan about 3% of H1N1 viruses, but no H3N2 or type B viruses, possessed the known oseltamivir-resistant NA mutation at position 274, no resistant viruses were found during the 2006–2007 season [35]. In the 2007–2008 season, 1.5%–2.6% of H1N1 viruses, which predominantly circulated in Japan, exhibited oseltamivir resistance [36–38], compared with 67% oseltamivir resistance among H1N1 viruses isolated in Norway in November of 2007 [15]. However, Matsuzaki et al showed that all H1N1 viruses isolated and tested in Japan during the 2008–2009 season were oseltamivir resistant [38], whereas no oseltamivir-resistant H3N2 or type B viruses were reported.

Although many oseltamivir-resistant viruses have been isolated globally, reports of clinical isolates resistant to zanamivir are quite limited. However, whether this is due to the limited use of zanamivir or to a property of the drug is unknown. We investigated the frequency of drug-resistant viruses in seasonal influenza virus-infected children treated with either oseltamivir or zanamivir, by collecting clinical specimens during the 2005–2006, 2006–2007, 2007–2008, and 2008–2009 influenza seasons and analyzing them for the presence of drug-resistant viruses. Our results indicate that zanamivir is superior to oseltamivir

with respect to emergence of drug-resistant viruses during therapy. Furthermore, we revealed that the use of zanamivir shortens the virus-shedding period in comparison with the use of oseltamivir.

METHODS

Patients and Samples

All patients tested positive for influenza using the influenza rapid diagnosis kit ESPLINE Influenza A & B-N (Fujirebio). Clinical specimens (throat or nasal swab) collected from a total of 144 pediatric patients who accessed the pediatric service at 4 hospitals (Eiju Hospital, Tokyo; and Keiyu Hospital and Zama and Ichikawa Children's Clinics, Kanagawa) in Japan during the 2005–2006, 2006–2007, 2007–2008, and 2008–2009 influenza seasons were subjected to virological analyses. All patients were treated with either oseltamivir (4mg/kg daily in divided doses twice a day for 5 days) or zanamivir (20 mg daily in divided doses twice a day for 5 days), beginning 48 h or less after onset of symptoms. The choice of NA inhibitors was based on patients' or parents' wishes or patients' clinical manifestations (eg, vomiting or wheezing). No other antiviral drugs were used. None of these patients was immunocompromised, was receiving corticosteroids or immunosuppressive drugs, or had a risk factor contraindicating the use of oseltamivir or zanamivir. All enrolled patients recovered without hospitalization.

We collected clinical specimens from each patient at 2 or 3 time points: at the initial visit (on day 1) just before drug administration and on day 3–4 and/or on day 5–7 after beginning drug treatment. From 72 patients treated with oseltamivir, 60 “second” and 33 “second or third” specimens were collected on day 3–4 and day 5–7, respectively. From 72 patients treated with zanamivir, 50 “second” and 33 “second or third” specimens were collected on day 3–4 and day 5–7, respectively. Oral informed consent was obtained from the parents of all patients. Our research protocol was approved by the ethics committee of each hospital and by the Research Ethics Review Committee of the Institute of Medical Science of the University of Tokyo (approval no. 20-46-1224).

Virus Isolation and Viral Genome Sequencing

The HA subtype of the viruses in the specimens was determined by means of conventional HA inhibition assay. Viral RNA was extracted directly from the collected specimens with the QIAamp Viral Mini kit (QIAGEN) and reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA products were used as templates to amplify NA and HA (for only isolates possessing oseltamivir-resistant NA mutations) genes by polymerase chain reaction (PCR) with Phusion High-Fidelity DNA polymerase (Finnzymes). We cloned the PCR products into the pCR-Blunt

Table 1. Characteristics of Pediatric Influenza Patients Studied

Parameter	Patients treated with oseltamivir (n = 72)	Patients treated with zanamivir (n = 72)	P
Type or subtype of viruses isolated from patients			.41
H1N1	21	26	
H3N2	39	31	
Type B	12	15	
Age, years			.11
Mean ± SD	7.7 ± 2.5	8.5 ± 2.8	
Range	4–15	4–15	
Sex, no. male /no. female	38/34	36/36	.86
No. (%) of patients with vaccination history	30 (42)	27 (38)	.61

II-TOPO vector (Invitrogen) and determined the entire nucleotide sequence of HA and NA genes from at least 12 clones of each sample with the ABI PRISM 3100 and 3130xl Genetic Analyzer (Applied Biosystems). The nucleotide sequence of primers used for reverse transcription, PCR, and viral genome sequencing is available on request. The specimens were also subjected to virus isolation and titration in Madin-Darby canine kidney cells, which were maintained in Eagle's minimal essential medium supplemented with 5% newborn calf serum (Sigma) and cultured at 37°C in 5% CO₂. For the neuraminidase inhibition assay (see below), plaque-purified viruses were used.

Neuraminidase Inhibition Assay

The sensitivity of influenza viruses to NA inhibitors was assessed with a neuraminidase inhibition assay [19, 32] using 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA; Sigma) as the fluorescent substrate. We incubated 10 μ L of diluted virus (containing 800–1200 fluorescence units of MUNANA) at 37°C for 30 min with an equal volume (0.01 nM to 10 mM) of the NA inhibitor oseltamivir carboxylate (the active form of oseltamivir; Roche Products) or zanamivir (GlaxoSmithKline) in calcium-MES buffer (33 mM 2-[N-morpholino]ethanesulfonic acid, 4 mM CaCl₂; pH, 6.0). Then, 30 μ L of MUNANA (0.1 mM) was added to the mixtures, followed by additional incubation at 37°C for 60 min. We stopped the viral NA-MUNANA reaction by adding 150 μ L of NaOH (0.1 M) in 80% ethanol (pH, 10.0) and measured fluorescence at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The median inhibitory concentration (IC₅₀) of NA inhibitors was determined by extrapolating the relationship between the concentration of inhibitor and the percentage of fluorescence inhibition.

Phylogenetic Analysis

NA gene sequences of human H1N1 and H3N2 viruses isolated during the period 2003–2009 were downloaded from the Influenza Research Database. All sequences were assembled and edited using BioEdit 7 software. Phylogenetic analysis was performed with the neighbor-joining method by using the Kimura 2-parameter nucleotide substitution model in MEGA 5 software [40]. Bootstrap values were calculated from 1000 replicates. Trees were rooted to A/New Caledonia/20/1999 (H1N1) and A/Wyoming/3/2003 (H3N2) for H1N1 and H3N2 viruses, respectively.

Statistical Analysis

Baseline data (measurements before drug administration) were compared with paired data by using the χ^2 test or Fisher exact test for categorical variables. To compare the data before and after drug administration, the χ^2 test was used for the virus isolation rate and the Fisher exact test was used for the frequency. The analyses were performed using SPSS 11.0 J (SPSS) for Windows. *P* values of <.05 were considered to be statistically significant.

RESULTS

Patient Information

We treated 144 pediatric influenza patients, 72 with oseltamivir and 72 with zanamivir. All enrolled patients exhibited fever (temperature, >37.5°C) for <5 days and relatively mild illness and therefore did not require hospitalization. There was no statistically significant difference between oseltamivir- and zanamivir-treated patient groups in terms of type or subtype of virus isolates, age distribution, sex, vaccination history (Table 1 and Supplementary Table S1), or clinical course (Supplementary Table S2), with the exception of body temperature at the first visit: the mean body temperature of zanamivir-treated patients was significantly higher than that of oseltamivir-treated patients (*P* = .01).

Effect of NA Inhibitors on Virus-Shedding Period

We first compared the effect of the 2 NA inhibitors on the virus-shedding period (Supplementary Table S3). Influenza viruses were isolated from 41 (68%) of 60 samples and 25 (50%) of 50 samples of the specimens that were collected on day 3–4 from the patients treated with oseltamivir and zanamivir, respectively, whereas 23 (69%) of 33 samples and 13 (39%) of 33 samples of the specimens collected on day 5–7 from the oseltamivir- and zanamivir-treated patients, respectively, contained infectious viruses. Although no statistically significant difference was found in virus isolation rate when specimens collected on day 3–4 from the oseltamivir-treated patients and zanamivir-treated patients were compared, the virus isolation rate from the specimens collected on day 5–7 was significantly higher in the

Table 2. Summary of Influenza Viruses Possessing a Neuraminidase (NA) Substitution

Virus ID	Amino acid substitution in NA	Subtype of virus	Age of patient, years	Date of sampling (day after beginning of treatment)	Proportion of viruses with substituted NA, % ^a	IC ₅₀ for oseltamivir, ^b nM	
						Parental virus	Mutant virus
1	Arg292Lys	H3N2	5	3	0	0.21	33,390
				5	25	0.21	33,390
2	Arg292Lys	H3N2	9	3	0	NT ^c	NT
				5	50	NT ^c	NT
3	Arg292Lys	H3N2	13	4	8	NT	NT
4	His274Tyr	H1N1	4	5	8	1.63	1115
				7	75	1.63	1115
5	His274Tyr	H1N1	9	4	16	NT	NT
6	Glu119Val	H3N2	5	4	41	0.43	230

^a For each virus isolate, we analyzed the nucleotide sequences of the NA genes of at least 12 molecular clones and calculated the proportion of cDNA clones encoding substituted NAs.

^b Median inhibitory concentration (IC₅₀) values are the mean of duplicate reactions.

^c NT indicates samples not tested because infectious viruses were not isolated from the corresponding specimens.

oseltamivir-treated group than in the zanamivir-treated group ($P = .008$). These results suggest that zanamivir decreases virus shedding (and thus shortens the virus-shedding period) more efficiently than oseltamivir does.

Frequency of Drug-Resistant Viruses

To detect an amino acid mutation that confers oseltamivir and zanamivir resistance to viruses, we sequenced the viral NA gene in all specimens collected from all enrolled patients. By comparing the deduced amino acid sequences of viruses collected before drug administration (on day 1), in which we found no amino acid known to confer NA inhibitor resistance to viruses, with those obtained at later time points, we detected NA substitutions in specimens collected from 6 oseltamivir-treated patients (8.3%) (Table 2). With regard to amino acid sequences of HA that were obtained only for viruses possessing NA substitutions, no difference was detected between viruses isolated before or after drug treatment.

Among the 6 NAs with a substitution, 3 N2 NAs possessed an arginine-to-lysine substitution at position 292 (Arg292Lys), 2 N1 NAs possessed a glutamic acid-to-valine substitution at position 119 (Glu119Val), and 1 N2 NA possessed a histidine-to-tyrosine substitution at position 274 (His274Tyr), all of which are known to confer oseltamivir resistance to viruses [10, 19, 22, 26]. On the basis of sequence analyses of more than 12 molecular clones of the NA gene for each sample, the proportions of viruses possessing the drug-resistant NAs were 50% or less in all the specimens tested, with the exception of 1 specimen collected on day 7, in which 75% of the cloned NA genes encoded a His274Tyr mutation. All of the resistant mutations were detected on or after day 4 of drug treatment (Table 2).

To assess oseltamivir sensitivity of the viruses possessing the substituted NAs (NA Arg292Lys, NA Glu119Val, and NA His274Tyr), we performed a neuraminidase inhibition assay [19,

32] using plaque-purified viruses isolated from 3 patients (Table 2). The IC₅₀ for oseltamivir of viruses possessing Arg292Lys, Glu119Val, and His274Tyr in NA were about 160,000-, 500-, and 680-fold higher than that of their parental viruses, respectively. We also tested the oseltamivir sensitivity of 3 isolates randomly picked from H1N1, H3N2, or type B viruses isolated from oseltamivir-treated patients during the 2005–2006, 2006–2007, 2007–2008, and 2008–2009 influenza seasons; the IC₅₀ for oseltamivir of these viruses without the resistant NA mutation was much lower than that of the representative oseltamivir-resistant viruses. These results confirm that the mutant NAs do indeed confer substantial resistance to viruses against oseltamivir, which is consistent with previous reports [10, 19, 22, 26].

Phylogenetic analysis of the NA gene of the H1N1 viruses revealed that the 2 resistant viruses (virus IDs 4 and 5) detected in this study fell within 2 distinct clades (Supplementary Figure 1A), as previously reported [41]. Virus ID 4 fell within clade 1, which is the clade of most viruses from the 2006–2007 season. In contrast, virus ID 5 fell within clade 2, which includes an A/Brisbane/59/2007-like virus (the vaccine strain for the 2008–2009 and 2009–2010 seasons). Currently circulating oseltamivir-resistant seasonal H1N1 viruses are also classified in this clade [42]. Although more than 95% of seasonal H1N1 viruses circulating after the 2008–2009 influenza season are resistant to oseltamivir, most of the seasonal H3N2 viruses remain susceptible [43]. Among the 4 oseltamivir-resistant H3N2 viruses detected in this study, virus ID 1 was shown to be an A/California/7/2004-like virus (the vaccine strain for the 2005–2006 season) by phylogenetic analysis of the NA gene for H3N2 viruses (Supplementary Figure 1A). Although both virus IDs 3 and 6 were classified as A/Wisconsin/67/2005-like viruses (the vaccine strain for the 2007–2008 season), they were not closely related to each other. Virus ID 2 fell as an intermediate between these

2 vaccine strains. These results suggest that the oseltamivir-resistant viruses isolated in this study emerged independently under drug-selective pressure through oseltamivir treatment.

In contrast to the oseltamivir-treated group, no NA substitutions were detected in viruses isolated from zanamivir-treated patients ($P = .03$). These results reveal that the frequency of viruses resistant to zanamivir is significantly lower than the frequency of those resistant to oseltamivir.

DISCUSSION

In this study, clinical specimens were collected from 2 statistically similar groups of pediatric influenza patients, one treated with oseltamivir and the other with zanamivir (Table 1). We then compared the frequency of seasonal influenza viruses resistant to oseltamivir or zanamivir in these patients (Table 2). Our results reveal that zanamivir has a substantial advantage over oseltamivir with regard to the emergence of resistant viruses in children. Furthermore, we found that zanamivir significantly shortened the virus-shedding period, compared with oseltamivir. Similar results were observed for zanamivir against H3N2, but not H1N1, influenza viruses in a previous study [12], although the findings were not statistically significant. Our data confirm the previous findings and suggest that zanamivir may be a superior therapeutic agent for pediatric patients who can be treated with inhaled drugs.

Oseltamivir-resistant viruses did not seem to cause increased or prolonged virus shedding (Supplementary Table S3) or increased severity or duration of illness (Supplementary Tables S3 and S4), although we did not find a statistically significant difference between oseltamivir-sensitive and oseltamivir-resistant viruses as a result of the limited number of cases of resistant virus infection.

Young children are immunologically immature compared with adults, allowing more persistent virus replication [44–49]—a dynamic that may be reflected by increased and sustained infection of young children during an influenza pandemic. The frequency of oseltamivir-resistant viruses in this study was 8.3% (6 of 72 patients treated with oseltamivir). Our results are comparable to those of previous studies indicating the higher frequency of oseltamivir-resistant viruses in children than in adults [19–21]. Taken together, oseltamivir-resistant variants may emerge more frequently in a pandemic situation in which the majority of patients are immunologically naive to the virus. In fact, oseltamivir-resistant swine-origin 2009 pandemic (H1N1) viruses have been isolated from patients treated with the drug and from those untreated [50], although sustained person-to-person transmission has not been reported thus far. However, studies have revealed that zanamivir is efficacious against oseltamivir-resistant isolates in vitro [18, 26, 51]. Furthermore, the efficacy of zanamivir is comparable to that of oseltamivir in both pediatric [12]

and adult [52] populations. Therefore, zanamivir may play an important role in the treatment of influenza.

Supplementary Material

Supplementary materials are available at Clinical Infectious Diseases online (http://www.oxfordjournals.org/our_journals/cid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

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