Resistant influenza A viruses in children treated with oseltamivir: descriptive study

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Summary

Background **Oseltamivir is an effective inhibitor of influenza virus neuraminidase. Although viruses resistant to oseltamivir emerge less frequently than those resistant to amantadine or rimantadine, information on oseltamivir-resistant viruses arising during clinical use of the drug in children is limited. Our aim was to investigate oseltamivir resistance in a group of children treated for influenza.**

Methods **We analysed influenza A viruses (H3N2) collected from 50 children before and during treatment with oseltamivir. We sequenced the genes for neuraminidase and haemagglutinin and studied the mutant neuraminidases for their sensitivity to oseltamivir carboxylate.**

Findings **We found neuraminidase mutations in viruses from nine patients (18%), six of whom had mutations at position 292 (Arg292Lys) and two at position 119 (Glu119Val), which are known to confer resistance to neuraminidase inhibitors. We also identified another mutation (Asn294Ser) in one patient. Sensitivity testing to oseltamivir carboxylate revealed that the neuraminidases of viruses that have an Arg292Lys, Glu119Val, or Asn294Ser mutation were about 10^4–10^5-fold, 500-fold, or 300-fold more resistant than their pretreatment neuraminidases, respectively. Oseltamivir-resistant viruses were first detected at day 4 of treatment and on each successive day of the study. More than 10^3 infectious units per mL of virus were detected in some of the patients who did not shed drug-resistant viruses, even after 5 days of treatment.**

Interpretation **Oseltamivir-resistant mutants in children being treated for influenza with oseltamivir arise more frequently than previously reported. Furthermore, children can be a source of viral transmission, even after 5 days of treatment with oseltamivir.**

Introduction

Influenza viruses continue to cause an unacceptable number of deaths and substantial economic losses worldwide. The last pandemic of influenza was in 1968, but a new pandemic virus will certainly arise. In addition to vaccines, two classes of antiviral agents have been used to treat influenza—the M2 ion channel inhibitors and the neuraminidase inhibitors. The M2 inhibitors amantadine and rimantadine inhibit influenza A viruses but drug-resistant mutants are present in about 30% of treated patients; resistant variants are genetically stable and can be transmitted from person to person.[1] Furthermore, we have shown[2] that the frequency of resistant viruses in children admitted and treated with amantadine could be as high as 80%.

Over the past few years, inhibitors of the viral enzyme neuraminidase have been used to treat influenza in clinical settings.[3] Whereas the viral haemagglutinin binds to cell-surface sialyloligosaccharide receptors and initiates virus infection, the enzymatic activity of neuraminidase sialidase removes sialic acids from the oligosaccharides, promoting the release of viruses from infected cells.[3] The importance of the neuraminidase in viral replication has made it an attractive target for the development of effective antiviral drugs. Knowledge of the conserved active sites for type A and type B neuraminidase molecules[4–6] led to the production of two specific and potent neuraminidase inhibitors, namely inhaled zanamivir and oral oseltamivir, which are therapeutically useful in various clinical situations.[7,8] As with any antiviral compound, the therapeutic importance of the neuraminidase inhibitors can be compromised by the emergence of drug-resistant mutants. In cell culture with a neuraminidase inhibitor, mutations are introduced into the surface proteins of influenza viruses.[9–11] Although such inhibitors act mainly on neuraminidase, viruses with haemagglutinin substitutions are usually selected first.[12] These mutations reduce the sialic acid-binding activity of the haemagglutinin and the virus becomes less dependent on the sialidase activity of the neuraminidase, whereas subsequent neuraminidase mutations render the virus fully drug resistant. Most of the neuraminidase mutations detected so far result in low or unstable neuraminidase activity.[10,13–15]

The neuraminidase substitutions in drug-resistant viruses include aminoacid residues 119, 152, 274, and 292 of the enzyme's active centre (N2 numbering system).[3,15] The arginine to lysine mutation (Arg292Lys) is the most typical substitution noted in N2-containing viruses isolated after treatment with oseltamivir in vivo and in vitro.[14–16] Induction of neuraminidase inhibitor resistance needs multiple passages in cell culture, by contrast with amantadine resistance, which typically

Panel: Primers used in the study

A-U12
5′ AGCAAAAGCAGG 3′

M13R
5′ CAGGAAACAGCTATGAC 3′

M13F-20
5′ GTAAAACGACGGCCAG 3′

H3N2-HA-1F
5′ CAATTGGACTGGAGTCGCTC 3′

H3N2-HA-2R
5′ CAGGGCAACAAGAAGCTCCG 3′

H3N2-NA-1F
5′ GGACAGGGAACAACACTAAACAACAGGC 3′

H3N2-NA-2R
5′ GGGTGTGTCTCCAACAAGTCTGAGCAC 3′

needs only one passage.[14,17,18] Thus, the low frequency of oseltamivir resistance in controlled clinical trials, about 4% for children aged 1–12 years and 0·4–1% for adults,[1,19,20] is not surprising. Our aim was to investigate such resistance in a group of children treated for influenza in Japan.

Methods

Patients

We obtained samples for influenza virus analysis from patients admitted to hospital and from outpatients during February to March, 2002 (Nippon Kokan Hospital), and January to February, 2003 (Keiyu Hospital, Kawasaki Municipal Hospital, Isehara Kyodo Hospital), all of whom were treated with oseltamivir. Treatment with oseltamivir (4 mg/kg daily in divided doses twice a day, for 2 [patient number 26], 3 [patients 27, 49], 4 [patients 16, 20], or 5 days [all remaining patients]) was not started until influenza infection was confirmed but always began within 48 h after onset of symptoms. We confirmed that patients aged younger

	Age (years [y], months [m])	Duration of hospital stay (days)	Days of fever before treatment	Total days of fever	Days of oseltamivir treatment	Number of influenza immunisations*	Underlying illness	Complications
Patient								
1	11 m	6	1	2	5	–	–	–
2	1 y 2 m	6	1	3	5	–	–	Bronchitis, febrile convulsion
3	1 y 3 m	6	1	4	5	–	–	Laryngitis
4	2 y 10 m	7	1	3	5	–	Bronchial asthma	Bronchitis, asthma attack
5	11m	8	1	5	5	–	–	–
6	1 y 6 m	6	1	3	5	–	–	–
7	1 y 10 m	11	2	3	5	–	–	Bronchitis
8	1 y 9 m	19	0	6	5	–	–	Bronchitis, otitis media, febrile convulsion
9	1 y	7	1	3	5	–	–	Pneumonia
10	1 y 3 m	8	2	7	5	–	–	Febrile convulsion
11	3 y	Outpatient	1	5	5	–	–	–
12	11 y	Outpatient	1	1	5	–	–	–
13	1 y	Outpatient	1	2	5	–	–	–
14	2 y	Outpatient	2	2	5	2	–	–
15	2 y	Outpatient	1	4	5	–	–	–
16	10 y	Outpatient	0	3	4	–	–	–
17	2 y	Outpatient	1	1	5	2	–	–
18	6 m	15	0	2	5	–	Urinary tract infection	–
19	3 m	3	0	2	5	–	–	–
20	1 y	9	1	4	4	–	–	Febrile convulsion
21	2 m	6	1	3	5	–	–	–
22	7 m	5	1	4	5	1	–	–
23	1 y	6	2	3	5	–	–	Febrile convulsion
24	13 y 6 m	6	0	3	5	–	Atopic dermatitis	Febrile convulsion
25	2 y	6	1	2	5	–	–	Febrile convulsion
26	2 y	Outpatient	1	6	2	–	–	–
27	14 y	Outpatient	1	2	3	2	Bronchial asthma	–
28	13 y	8	1	3	5	–	Bronchial asthma	Pneumonia
29	2 y 7 m	6	0	2	5	–	–	–
30	3 y 5 m	4	0	2	5	–	–	Febrile convulsion
31	5 y	Outpatient	1	2	5	–	–	–
32	2 y	8	0	4	5	2	–	Febrile convulsion
33	1 y	5	1	2	5	–	–	Febrile convulsion
34–50†	4 y 5 m	6·8	1·1	2·4	4·9	1	–	–

*For the season tested. †Virus was not present for patients 34–50 post-treatment. Information for these patients is therefore shown as an average. Six patients were admitted, two patients immunised once, and one patient had bronchitis.

Table 1: **Information on H3N2 influenza A virus-infected patients from whom samples were taken**

than 1 year had no specific risk factors prohibiting treatment, and their conditions were closely monitored for adverse drug reactions. We obtained oral informed consent from the parents of all patients.

Procedures

We took nasal swabs, nasal aspirates, or throat swabs from all patients at least twice during the study (at the initial visit to a doctor and at least once during treatment). We ascertained viral subtypes by conventional haemagglutinin and neuraminidase inhibition assays. We used the samples for direct RNA extraction or for isolation of influenza virus on Madin-Darby canine kidney (MDCK) cells. Influenza virus in each specimen was titrated by plaque assay with MDCK cells.[21]

With respect to sequence analysis of viral isolates, we extracted viral RNA from the specimens directly or from virus in cell-culture fluids by use of an RNA extraction kit (ISOGEN-LS, Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. For the reverse-transcription reactions, we used the A-U12 primer (panel), which is complementary to the 3′ end of the viral RNA and reverse transcriptase (SUPERSCRIPT II or III; Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. We then used the cDNA products to amplify haemagglutinin and neuraminidase genes by a standard PCR method (Pwo DNA polymerase; Roche, Basel, Switzerland). The thermocycler programme was as follows: initial denaturation at 94°C for 2 min 30 s, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 10 min. We ran the resultant PCR products on a 1% agarose gel and purified them with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).[2]

We ligated the purified PCR products into the pCR-Blunt II-TOPO vector (Invitrogen) and transformed them into TOP10 cells (Invitrogen). We cultured positive clones in Luria broth containing 50 mg/L kanamycin and incubated them overnight at 37°C in a shaking incubator. We pelleted the bacterial culture by centrifugation, and extracted plasmid DNA for sequencing with the MagExtractor-plasmid system (TOYOBO, Osaka, Japan). We analysed haemagglutinin and neuraminidase gene sequences with the Applied Biosystem 3100 Auto Sequencer (Foster City, CA, USA) by use of cycle sequencing dye terminator chemistry (Perkin Elmer, Boston, MA, USA) with M13R, M13F-20, haemagglutinin-specific, or neuraminidase-specific primers (panel). We sequenced at least ten cDNA clones of the haemagglutinin and neuraminidase genes for each sample.

We isolated influenza viruses from clinical specimens and propagated them for a maximum of three passages in MDCK cells before use in drug-sensitivity and other tests. To obtain mutant viruses with a particular mutation, we picked viral plaques and propagated the viruses and confirmed their sequences.

	Day samples containing mutant viruses were taken	Mutations found in neuraminidase		Mutations found in haemagglutinin	
		Mutation	Proportion of virus population (%)*	Mutation	Proportion of virus population (%)*
Patient					
4	6	Arg292Lys	80	–	–
5	4	Arg292Lys	60	–	–
9	6	Arg292Lys	90	Ser262Asn	55
10	7	Asn294Ser	100	–	–
11	5	Arg292Lys	100	–	–
13	5	Glu119Val	92	–	–
14	5	Glu119Val	29	–	–
23	5	Arg292Lys	91	–	–
25	4	Arg292Lys	100	–	–

*Ascertained by analysis of at least ten molecular clones of the genes.

Table 2: **Aminoacid mutations in viruses isolated from patients treated with oseltamivir**

Oseltamivir carboxylate (GS4071; Roche Products) is the active compound of the ethyl ester prodrug oseltamivir phosphate. We did plaque reduction assays in the presence of GS4071 in an agar overlay on MDCK cells. Isolates were first titrated on MDCK monolayers without the compound to ascertain plaque-forming units (PFU) per mL of viral stock. We infected confluent monolayers of MDCK cells in six-well plates with about 50 PFU of virus for 1 h and then incubated them with an agar overlay containing dilutions of the compound to be tested. We incubated plates for 2 days, removed the overlays, and stained the monolayers with 0·1% crystal violet in 20% methanol.

We assessed the sensitivity of the viral neuraminidase to oseltamivir carboxylate with a neuraminidase enzyme inhibition assay based on the method described by Gubareva and colleagues.[22,23] We used methylumbelliferyl-N-acetylneuraminic acid (MUNANA, Sigma, St Louis, MO, USA), at a final concentration of 0·1 mmol/L, as a fluorescent substrate. We used virus dilutions with 800–1200 fluorescence units in the neuraminidase inhibition assay. We mixed diluted virus and the drug (0·01 nmol/L to 1 mmol/L) in 33 mmol/L 2-(N-morpholino)ethanesulfonic acid (pH 6·0), containing 4 mmol/L calcium chloride, and incubated this mixture for 30 min at 37°C. We then added the substrate. After 1 h at 37°C, the reaction was stopped by adding 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. We ascertained the relation between the concentration of inhibitor and the proportion of fluorescence inhibition and obtained IC_{50} values from extrapolation of those findings.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full

	Neuraminidase mutation*	IC_{50} of parent virus (nmol/L)†	Reduction in drug sensitivity (fold)‡
Patient			
4	Arg292Lys	0·18	110 000
9	Arg292Lys	0·28	45 400
10	Asn294Ser	0·38	280
13	Glu119Val	0·46	520
23	Arg292Lys	0·28	55 000
25	Arg292Lys	0·46	28 000

*Arg292Lys and Glu119Val mutants from patients 5, 11, and 14 were not tested. †Data are the means of duplicate reactions. ‡Comparison between viruses with a neuraminidase mutation and parent virus.

Table 3: **Effect of aminoacid mutations on neuraminidase sensitivity to GS4071**

access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We obtained samples for influenza virus analysis from 14 patients admitted and from one outpatient during February to March, 2002, and from 15 patients admitted and 20 outpatients during January to February, 2003. Patients ranged in age from 2 months to 15·8 years (median 3·7), and 80% were younger than age 5 years (table 1). Only seven (14%) of 50 individuals had received influenza vaccine. Although several children had pre-existing conditions, none was immunocompromised or receiving corticosteroids or immunosuppressive drugs. Their influenza symptoms consisted of high-grade fever, cough, and other influenza-like complaints. Fever lasted a median of 2·9 days; five (10%) had fever that persisted for 5 days or longer.

After treatment with oseltamivir, we detected the haemagglutinin and neuraminidase genes by RT-PCR in 33 of the 50 patients sampled, indicating that the virus had been cleared from the remaining patients by the time of the second sampling. To detect oseltamivir-resistant viruses, we extracted viral RNA directly from the specimens of 30 patients or from virus in cell-culture fluids of the remaining three (patients 1, 3, and 5) and then cloned the haemagglutinin and neuraminidase genes. This approach was taken because in a previous study of amantadine-resistant influenza viruses, we noted that drug-resistant and drug-sensitive viruses could coexist in the same patient, so that direct sequencing of clinical specimens might not detect minor drug-resistant populations of virus, especially when multiple resistant viruses coexist.[2] Based on neuraminidase sequencing, no pretreatment isolates contained known aminoacid residues associated with oseltamivir resistance.

By comparing the deduced aminoacid sequences for the neuraminidases of viruses isolated from samples obtained during the initial visit to the doctor with those ascertained at later times, we identified a neuraminidase mutation in viruses isolated from nine (18%) of the 50 oseltamivir-treated patients (table 2). In eight cases, the mutations were known to confer resistance to neuraminidase inhibitors: six Arg292Lys and two

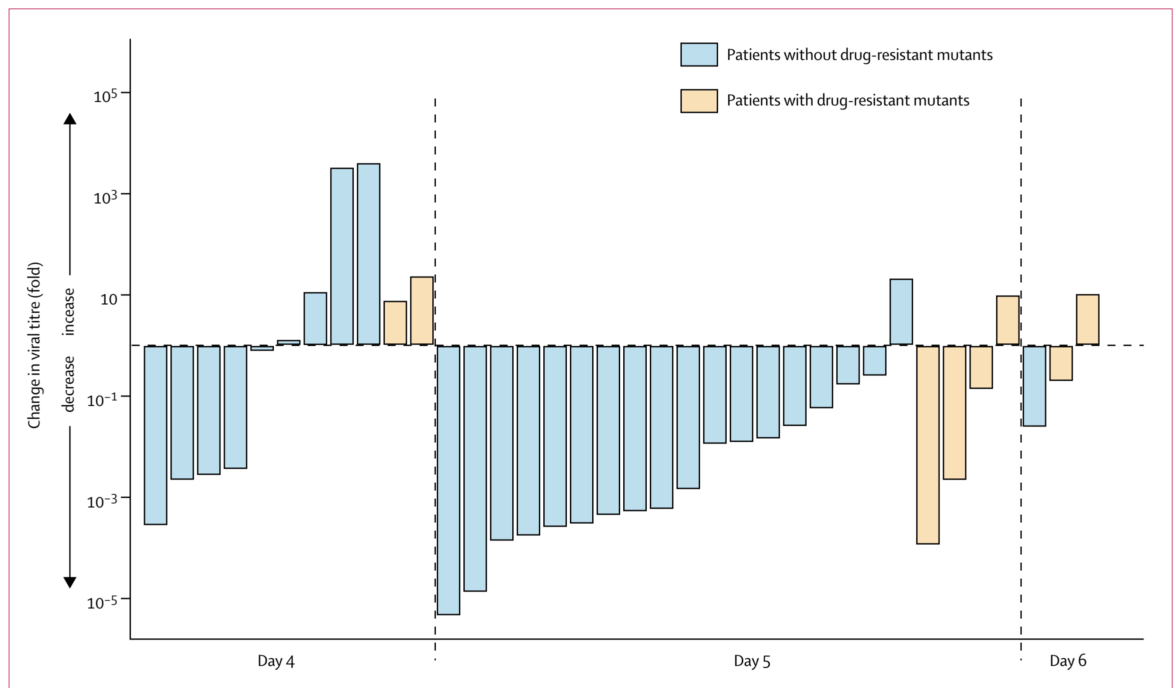

Figure: **Change in virus titres in patients treated with oseltamivir**
Virus titres of paired samples from individual patients (one obtained before and another after treatment [day 4, 5, 6]) were compared.

Glu119Val mutations (table 2). In one child (patient 10), we detected an Asn294Ser mutation, which has not been associated with neuraminidase inhibitor resistance. The viruses with mutant neuraminidase genes coexisted with wild-type viruses in six of the nine cases and had fully replaced wild-type viruses in samples from three children. In one patient, the wild-type virus was dominant (patient 14, 29% of the neuraminidase clones had a Glu119Val mutation, whereas the remaining 71% harboured the wild-type neuraminidase). We detected a haemagglutinin mutation, Ser262Asn, in only one child (patient 9, who also had an Arg292Lys mutation in neuraminidase).

For viruses with neuraminidase substitutions (Arg292Lys, Glu119Val, and Asn294Ser) and the corresponding pretreatment viruses, we did in-vitro susceptibility testing by neuraminidase inhibition and plaque reduction assays with plaque-purified clones (table 3). The neuraminidase possessing the Arg292Lys mutation was about 10^4–10^5-fold more resistant to GS4071 than was the pretreatment neuraminidase, whereas the neuraminidase with the Glu119Val mutation was about 500-fold more resistant than its pretreatment counterpart, in agreement with previous reports.[10,16,24] The Asn294Ser mutation conferred about 300-fold more resistance to oseltamivir than did its pretreatment molecule (table 3).

We also did plaque reduction assays with MDCK cells in the presence of oseltamivir carboxylate, but the drug did not inhibit the growth of parental or mutant viruses as previously reported.[22,25,26]

To ascertain the temporal pattern of emergence of oseltamivir-resistant viruses, we analysed viral sequences of specimens before and on days 3–8 of drug administration. None of the samples collected before or on day 3 of treatment contained viruses with a drug-resistant neuraminidase. We detected drug-resistant mutants as early as 4 days after start of treatment, and positive specimens persisted on each succeeding day of testing through day 7. In two patients for whom we were able to obtain day 3 and day 5 samples, we found the Arg292Lys neuraminidase mutant on day 5 but not on day 3.

We assessed the factors potentially associated with emergence of resistance in 50 patients. In those aged younger than 1 year, 1 year, 2–6 years, and 7 years or older, we noted resistant variants in one of nine, four of 12, four of 22, and none of seven, respectively. We detected drug-resistant mutants at similar frequencies in children admitted to hospital (6 of 29) and in those managed as outpatients (3 of 21). One resistant variant was detected in the seven children who had received influenza vaccine, compared with eight of the 43 not receiving vaccine.

To identify whether drug treatment affects virus shedding in oseltamivir-treated patients, we assessed viral titres in samples obtained from 50 patients before and during antiviral therapy. Of 50 patients, five were influenza A positive by a rapid diagnostic test and were RT-PCR positive for H3 genes at the time of initial visit to the doctor, but virus was not isolated at the initial visit or after drug treatment. Thus, only samples from 45 patients underwent subsequent analysis for changes in viral titres on drug treatment. On day 4 of treatment, four of nine patients without drug-resistant mutants were shedding the same or more virus than they did on initiation of therapy, and two children with mutant viruses showed an increase in viral titre (figure, table 4). On day 5 or 6, the level of virus shedding was reduced in 18 of 19 patients without resistant viruses and in four of six with resistant viruses. Even after 5 days of treatment, more than 10^3 infectious units per mL of virus were detected in samples from some patients, irrespective of whether they shed resistant viruses (table 4).

	Patients without oseltamivir-resistant mutants			Patients with oseltamivir-resistant mutants		
	Patient number (age)	Viral titre (PFU per mL)		Patient number (age)	Viral titre (PFU per mL)	
		Pre	Post		Pre	Post
Day specimen obtained*						
3	31 (5 y)	3×10^{4}	7×10^{2}			
	32 (2 y)	6×10^{3}	<10			
	33 (1 y)	2×10^{3}	$3\cdot1\times10^{3}$			
	41(3 y 10 m)	10	<10			
	45(6 m)	$1\cdot8\times10^{5}$	<10			
4	6 (1 y 6 m)	$2\cdot8\times10^{2}$	3×10^{3}	5 (11 m)	3×10	7×10^{2}
	7 (1 y 10 m)	<10	3×10^{3}	25 (2 y)	$3\cdot6\times10^{3}$	$2\cdot7\times10^{4}$
	12 (11 y)	$2\cdot4\times10^{4}$	6×10			
	16(10 y)	3×10^{3}	10			
	19 (3 m)	5×10^{6}	$1\cdot5\times10^{3}$			
	26 (2 y)	$4\cdot8\times10^{5}$	$3\cdot3\times10^{5}$			
	29 (2 y 7 m)	1×10^{4}	$1\cdot2\times10^{4}$			
	30 (3 y 5 m)	2×10^{2}	5×10^{5}			
	46(4 y 3 m)	$2\cdot3\times10^{2}$	<10			
5	1 (11 m)	$1\cdot5\times10^{5}$	5×10^{4}	11 (3 y)	$2\cdot7\times10^{5}$	3×10
	2 (1 y 2 m)	5×10^{5}	$1\cdot5\times10^{4}$	13 (1 y)	$4\cdot5\times10^{3}$	$3\cdot9\times10^{4}$
	3 (1 y 3 m)	8×10^{5}	1×10^{3}	14 (2 y)	$5\cdot6\times10^{3}$	10
	15 (2 y)	$9\cdot8\times10^{4}$	1×10^{3}	23 (1 y)	$1\cdot3\times10^{5}$	$1\cdot6\times10^{4}$
	17 (2 y)	$3\cdot2\times10^{5}$	2×10^{4}			
	18 (6 m)	$2\cdot1\times10^{5}$	3×10^{3}			
	20 (1 y)	$1\cdot2\times10^{6}$	$5\cdot5\times10^{2}$			
	22 (7 m)	$2\cdot3\times10^{3}$	$5\cdot2\times10^{2}$			
	24 (13 y 6 m)	$1\cdot3\times10^{3}$	3×10^{4}			
	27 (14 y)	$1\cdot3\times10^{5}$	2×10			
	28 (13 y)	4×10^{3}	$4\cdot8\times10^{2}$			
	36 (4 y 8 m)	2×10^{3}	<10			
	37 (2 y 4 m)	2×10^{5}	<10			
	39 (5 y 3 m)	$4\cdot7\times10^{3}$	<10			
	43 (2 y)	4×10^{3}	<10			
	44 (4 y 3 m)	$1\cdot7\times10^{3}$	<10			
	48 (3 y)	5×10^{4}	<10			
	50 (5 m)	6×10^{3}	<10			
6	21 (2 m)	$6\cdot5\times10^{3}$	$1\cdot5\times10^{2}$	4 (2 y 10 m)	2×10^{3}	2×10^{4}
				9 (1 y)	3×10^{5}	6×10^{4}
7	42 (1 y 3 m)	$3\cdot2\times10^{2}$	<10	10 (1 y 3 m)	4×10^{4}	<10
	49 (2 y)	$3\cdot5\times10^{4}$	<10			
8	8 (1 y 9 m)	5×10^{6}	5×10^{4}			

*Specimens obtained on day of initial doctor consultation (pre) and on days 3–8 thereafter (post).

Table 4: **Changes in viral titre before and after treatment with oseltamivir**

Discussion

The difficulty of generating viruses resistant to neuraminidase inhibitors in cell culture,[14] and the low frequency of such viruses in patients treated in clinical trials,[11,15,16] have led to the impression that the development of resistance is a minor difficulty compared with the emergence of amantadine or rimantadine resistant viruses. However, in our study, about a fifth of children developed resistance by day 4 or later during treatment with oseltamivir. Furthermore, just over a quarter of children who shed virus for 3 days or more had drug-resistant influenza viruses.

Our study population consisted entirely of children, a group in which the course of influenza is typically more protracted, with longer periods of virus shedding and higher virus titres than in adults. The results of a previous study[20] also indicated higher numbers of oseltamivir-resistant variants in treated children than in treated adults. In this study,[20] ten of 182 oseltamivir-treated ambulatory children aged 1–12 years had detectable resistant virus on day 4 or 6 after treatment. We noted that children with resistant variants had more protracted virus shedding, but our study design did not allow us to critically assess the relation between resistance emergence and clinical course. Whitley and colleagues[20] did not find evidence for worsening illness in the ten children with detectable resistant variants during treatment with oseltamivir, but further studies are needed.

We detected resistant viruses more often in young children, presumably experiencing their first or perhaps second influenza infection, than in older individuals. In this respect, our population of patients might be judged comparable with one experiencing pandemic influenza in the absence of pre-existing immunity to haemagglutinin and neuraminidase; however, in pandemics, most patients do have cellular immune memory to conserved internal proteins of influenza viruses, possibly reducing viral replication to some extent. Still, people infected with these viruses might shed them in greater amounts and for longer periods than during regular epidemics. Our results are, therefore, relevant to the control of influenza pandemics with neuraminidase inhibitors.

The high proportion of oseltamivir-resistant individuals in our study, by comparison with previous reports,[1,15,22] might be explained in part by our rigorous detection techniques. The molecular cloning of the neuraminidase genes from clinical specimens would identify viruses that might be overlooked in assays done on mixed populations of mutant and parental viruses. Indeed, when we applied similar techniques to the study of amantadine-treated children,[2] we detected resistant variants in 80% of children admitted and treated with amantadine for acute influenza.

Also, the virus might have replicated at a high level in the 29 children who were admitted for oseltamivir treatment, resulting in a higher frequency of resistant viruses. A third contributing factor could have been the drug doses used in young children in Japan—ie, oseltamivir 4 mg/kg daily—whereas in other countries, the drug is given according to weight groups (eg, ≤15 kg, 60 mg/day; 15–23 kg, 90 mg/day; 23–40 kg, 120 mg/day; >40 kg, 150 mg/day; in both the USA and Europe). Thus, for children weighing 15 kg or less, the oseltamivir dose would be lower in Japan than in other countries (40 mg/day for a child weighing 10 kg *vs* 60 mg/day) and might be suboptimum, contributing to a higher frequency of resistant viruses.

Results of previous work in murine and ferret models of influenza suggest that viruses resistant to neuraminidase inhibitors are less pathogenic and less transmissible than their pretreatment viruses.[27,28] However, these studies were done with drug-resistant viruses selected in cell culture with extra haemagglutinin mutations.[27,28] Indeed, neuraminidase inhibitor-resistant influenza viruses selected in vivo and without any additional haemagglutinin mutations, such as those described here, could retain their pathogenicity in people, emphasising the need to continue monitoring for the emergence and spread of these mutant viruses.

Neuraminidase inhibitors are highly effective antiviral compounds.[29] Thus, given that about 6 months or longer is needed to produce a new influenza vaccine,[30] these drugs, if available in sufficient amounts, could play an important part in the response to future pandemics. Our results suggest that a higher prevalence of resistant viruses should be expected during pandemic control efforts using neuraminidase inhibitors than during interpandemic episodes.

Contributors

M Kiso designed and did laboratory experiments, analysed data, and wrote the report. K Mitamura and K Kimura obtained clinical samples, coordinated work between clinicians and laboratories, and helped to write the report. Y Sakai-Tagawa and C Kawakami designed and did laboratory experiments and helped to write the report. K Shiraishi designed the laboratory experiments, analysed data, and helped to write the report. F G Hayden designed the study, analysed data, and helped to write the report. N Sugaya designed the clinical phase of the study, obtained clinical samples, and helped to write the report. Y Kawaoka had the idea for the project, outlines the design of the experiments, analysed data, and helped to write the report.

Conflict of interest statement

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