Selection of Influenza Virus Mutants in Experimentally Infected Volunteers Treated with Oseltamivir

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Volunteers experimentally infected with influenza A/Texas/36/91 (H1N1) virus and treated with the neuraminidase (NA) inhibitor oseltamivir were monitored for the emergence of drug-resistant variants. Two (4%) of 54 resistant viruses were detected by NA inhibition assay among last-day isolates recovered from 54 drug recipients. They bore a substitution His274Tyr in the NA. Hemagglutinin (HA) variants detected in the placebo group differed from the egg-adapted inoculum virus by virtue of amino acid substitutions at residues 137, 225, or both. These variants had a higher affinity for Neu5Ac(α2-6)Gal-containing receptors, which are characteristic of human respiratory epithelium, than for Neu5Ac(α2-3)Gal-containing receptors, which are typical of chicken egg allantoic membrane. Although appearing to be more sensitive to oseltamivir in humans, the variants with increased affinity for Neu5Ac(α2-6)Gal receptors were less sensitive than the Neu5Ac(α2-3)Gal-binding variants in Madin-Darby canine kidney cells. Thus, HA affinity for receptors is an essential feature of influenza virus susceptibility to NA inhibitors, both in cell culture and in humans.

Two inhibitors of influenza virus neuraminidase (NA)—zanamivir and oseltamivir—provide both antiviral effects and clinical benefits in humans with influenza [1]. In light of the experience with amantadine and rimantadine [2], a major concern in the use of any influenza antiviral drug is the emergence of resistant strains. Although the mechanisms of resistance to NA inhibitors have been studied extensively in vitro, little information is available regarding the emergence of resistant variants in vivo. In cell culture, the acquisition of resistance to NA inhibitors occurs in 2 steps: reduction of the virus's dependence on NA activity due to changes in the hemagglutinin (HA), followed by the acquisition of NA resistance due to changes in the enzyme itself [3–5]. Whether this 2-step mechanism occurs in humans is unclear.

Inhibitors of the viral NA mimic the structure of N-acetylneuraminic acid, a receptor determinant recognized by both influenza A and B viruses. The interaction of influenza viruses with N-acetylneuraminic acid–containing cellular receptors is mediated by both the HA and the NA surface glycoproteins. HA binding to receptors initiates viral infection, whereas the NA acts later to promote the release of virus from infected cells. Specifically, the NA cleaves a terminal N-acetylneuraminic acid residue from an oligosaccharide chain, thereby destroying the HA receptors and preventing the progeny virions from self-aggregating and binding to surface of infected cells [6]. The NA seems to prevent the entrapment of virus by sialic acid–containing inhibitors found in mucus secretions in the human respiratory tract [7].

The ability of influenza viruses to bind to N-acetylneuraminic acid depends on their recognition of either an α2-6 or α2-3 linkage connecting the acid to the adjacent oligosaccaride moiety. Depending on their host origin (human, avian, porcine, or equine), influenza viruses are shown to be distinctive in the recognition of this linkage [8–11]. Human viruses preferentially bind to and infect cells containing Neu5Ac(α2-6)Gal receptors, which are abundant on ciliated epithelial cells of the human respiratory tract [8, 12]. In contrast, the propagation of human viruses in embryonated chicken eggs consistently leads to the selection of receptor-binding variants with increased affinity for Neu5Ac(α2-3)Gal receptors on cells of the allantoic membrane ("egglike" HA variants) [13, 14]. Although viruses grown in Madin-Darby canine kidney (MDCK) cells, which express receptors with both types of linkages, do not readily acquire such

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affinity for Neu5Ac(α2-3)Gal-receptors, those recovered from chicken eggs do show a growth advantage in MDCK cells over viruses lacking egg-selected changes in the HA [15].

Virus resistance to NA inhibitors could develop from reduced HA affinity for cellular receptors because of mutations in its receptor binding site [16]. This NA-independent mechanism is supported by in vitro experiments showing that such changes facilitate the release of virus from infected cells, thereby decreasing the reliance of the virus on the NA function. An NA-dependent mechanism of resistance involves specific amino acid substitutions at residues forming the enzyme active site that render the enzyme resistant to specific inhibitors [17–21]. Mutations in the NA active site were reported in viruses that have been passaged many times in cell culture in the presence of an NA inhibitor and appear in both framework and catalytic site residues [1]. Although acquired changes did not necessarily impair virus growth in vitro, they often caused attenuated replication in animal models [17, 22, 23].

The aim of the present study was to investigate the potential for the emergence of oseltamivir-resistant variants in human volunteers treated with this NA inhibitor, after experimental infection with a specific influenza A (H1N1 subtype) virus. The antiviral effects of oseltamivir in the same study population were described elsewhere [24].

Methods

Compounds. The neuraminidase inhibitor oseltamivir carboxylate (GS4071) was provided by Gilead Sciences and was administered orally as the bioavailable prodrug oseltamivir phosphate (GS4104).

Study design. As described elsewhere [24], healthy adults susceptible to the challenge virus (serum HA inhibition antibody titers ≤1:8) were intranasally inoculated with ~10^6 $TCID_{50}$ of a safety-tested, egg-grown pool of influenza A/Texas/36/91 (H1N1) virus (provided by the National Institute of Allergy and Infectious Diseases). The subjects then were assigned randomly in a double-blinded manner to 1 of 5 treatment groups: placebo or oseltamivir twice daily at 20 mg, 100 mg, or 200 mg or once daily at 200 mg. Drug administration began 28 h after influenza virus inoculation and continued for 5 days. Nasal washes were collected for 8 days, and viruses were isolated with the use of MDCK cell monolayers. Frozen aliquots of samples that were positive on initial isolation were used to determine the $TCID_{50}$ per milliliter of sample in MDCK cells. Viruses isolated on the first and last days of shedding were passaged once or twice in MDCK cells and were stored at −70°C until further analyses.

Additional virologic measurements included the number of days of viral shedding after the start of treatment, virus titers in daily nasal washes, and estimates of the total virus load, based on calculating the area under the virus-titer curve (AUC) [24].

Plaque assay in MDCK cells. The susceptibility of virus isolates to oseltamivir was determined by plaque assay in confluent MDCK cell monolayers, as described elsewhere [25]. In brief, duplicate monolayers were inoculated with virus (25–60 pfu per well of a 6-well plate), were incubated at room temperature for 1 h, and were overlaid with Dulbecco-modified Eagle's medium (DMEM; Bio-Whittaker), supplemented with trypsin, agarose (0.6%), and oseltamivir. The final concentrations of drug incorporated in the overlay were 0.003, 0.03, 0.3, and 3 μ*M*. After 68 h of incubation at 34°C, the monolayers were fixed with 5% glutaraldehyde and were stained with 0.12% carbol fucsin after removal of the agar overlay. Estimates of viral sensitivity were based separately on reductions in plaque size and plaque number [26]. All plaques with the diameter of ≥0.5 mm were counted, and the concentration of drug that reduced the number of plaques by 50% (EC_{50}) was determined with dose-effect analysis software (Biosoft). EC_{50} values for reduction in plaque size were based on measurements of plaque diameters (10 plaques per monolayer). All experiments were performed in duplicate or triplicate, and the average EC_{50} values were calculated.

NA inhibition assay. The sensitivity of the viral NA to oseltamivir was evaluated with an NA enzyme inhibition assay (NI) based on the method of Potier et al. (1979) [27], with modifications. Methylumbelliferyl-N-acetylneuraminic acid (MUNANA, Sigma), at a final concentration of 0.1 m*M,* was used as a fluorescent substrate. Dilution of the virus and the drug (0.1 n*M*–1 μ*M*) in 33 m*M* 2-[*N*-morpholino]ethanesulfonic acid (pH 6.5) containing 4 m*M* $CaCl_2$ were mixed and incubated at room temperature, followed by addition of the substrate. The reaction was carried out at 37°C for 30 min and was stopped with the addition of 150 μL of 0.1*M* glycine buffer (pH 10.7) containing 25% ethanol. Fluorescence was quantified at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The IC_{50} was determined for the virus isolate on the last day of shedding and was compared with that of the challenge virus.

Receptor-binding properties. The receptor-binding activity of the viruses was characterized by assaying their ability to agglutinate erythrocytes predominantly bearing either Neu5Ac(α2-3)Gal- or Neu5Ac(α2-6)Gal-linked receptors [22] at 2 different temperatures (4°C and 37°C). An elevated temperature allows for better differentiation of binding properties of the viruses because viruses with reduced affinity of binding may produce hemagglutination at low temperature but often fail to agglutinate erythrocytes at elevated temperatures (37°C). Fifty microliters of 2-fold dilutions of virus was mixed with 50 μL of a 0.5% suspension of erythrocytes and was incubated in V-bottomed microtiter plates, and hemagglutination titers were determined after 45 min of incubation. Chicken erythrocytes were the source of cells predominantly carrying α2-3-linked receptors, whereas those with the α2-6 linkage were produced by treating human erythrocytes (O-type blood) with the NA of the Newcastle disease virus (kindly provided by A. Portner, St. Jude Children's Research Hospital, Memphis, TN), which selectively destroys Neu5Ac(α2-3)Gal-terminated receptors.

Sequence analysis of the HA and NA viral genes. Viral RNA was extracted from cell culture supernatants with the RNeasy kit (Qiagen). The synthetic oligonucleotide 5′-AGCAAAAGCAGG-3′ was used as a primer to generate cDNA with reverse transcriptase (RT; Gibco BRL). cDNA was amplified by standard polymerase chain reaction (PCR) method [22] and was purified with the Qiaquick PCR purification kit (Qiagen). Purified PCR products encoding the NA and HA1 subunits of the HA molecule were sequenced by use of Taq Dye Terminator chemistry, according to the

manufacturer's instructions (Applied Biosystems), and then were analyzed on an ABI 373 DNA sequencer (Applied Biosystems) at the Center of Biotechnology at the University of Virginia. Sequencer 4.0 software (Gene Codes Corporation) was used for the analysis and translation of nucleotide data.

A modified "nested" PCR procedure [28] was used to amplify partially the HA and NA genes of viruses present in the original nasal washes before their propagation in MDCK cells for direct sequence analysis.

Results

Subjects. Eighty adult healthy volunteers (18–40 years old) were inoculated intranasally with H1N1 virus, and 66 (79%) showed laboratory evidence of infection. Twelve of the infected volunteers were treated with placebo and 54 with oseltamivir.

NA susceptibility. The NA of the challenge virus was highly susceptible to oseltamivir inhibitor (mean IC_{50} value, 0.4 n*M*). To determine whether NA susceptibility was altered in response to treatment, we tested virus isolates recovered on the last day of shedding from all infected participants by the NI assay. Viruses recovered from 2 (4%) of the 54 oseltamivir recipients had IC_{50} values of 725 n*M* that were substantially (>1500-fold) increased. The median IC_{50} values for the last-day isolates from the either recipients of oseltamivir or placebo were 0.45 and 0.52 n*M,* respectively. Both resistant isolates were recovered from subjects receiving the highest dose of oseltamivir (200 mg once or twice a day). Both volunteers (5 and 35) had limited decreases in virus titers followed by virus rebound. They shed virus for >6 days (figure 1). Sequence analysis of the NA genes of the last-day isolates from these volunteers predicted an amino acid substitution (His→Tyr) at conserved residue 274 in the NA active site. Sequential nasal washes collected from one volunteer (35) were amplified by RT-PCR before virus propagation in MDCK cells. Sequence analysis of the products revealed that the NA substitution emerged after 84 h of treatment (figure 2).

Plaque size reduction in MDCK cells. Because virus resistance to the NA inhibitor could have emerged through an NA-independent mechanism [3, 16, 29], we also assessed the oseltamivir susceptibility of recovered viruses in cell culture by testing those isolates that were recovered from the 14 subjects, both placebo and oseltamivir recipients, who shed virus for >3 days after initiating treatment. The EC_{50} values for the challenge virus were <0.03 μM by plaque assay in MDCK cells, using reduction in either plaque size or plaque number as evaluation criteria. All last-day isolates, except the 2 showing oseltamivir resistance in the NA inhibition assay, had a drug-sensitive phenotype based on the reduction of plaque size ($EC_{50} < 0.03$ μM [table 1]). Hence, there was good agreement between NA resistance in NA inhibition assay and virus resistance in the plaque-size assay in MDCK cells.

We then assessed the drug-sensitivity phenotype of sequential virus isolates recovered from a drug recipient (35), selected because of virus resistant phenotype on the last day of shedding,

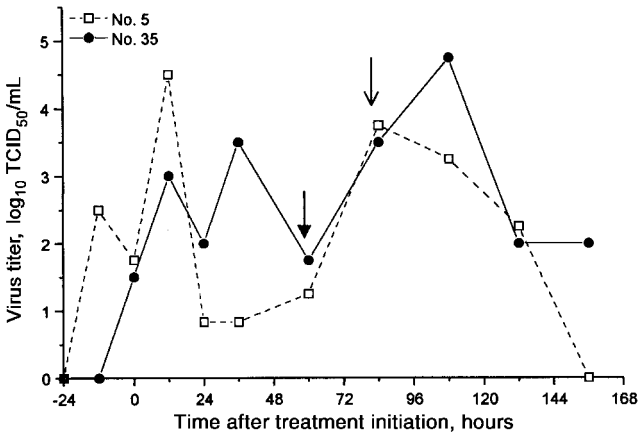

Figure 1. Virus titers in nasal washes after experimental A/Texas/36/91 (H1N1) infection and oseltamivir treatment of drug recipients 5 and 35. Virus inoculation preceded drug administration by 28 h. Titers were determined in Madin-Darby canine kidney (MDCK) cells. The solid-head arrow at 60 h of treatment indicates the time when resistance was detected by plaque assay of the virus, which was recovered from drug recipient 35 and was propagated twice in MDCK cells before testing. The open-head arrow at 84 h indicates when neuraminidase resistance was detected by sequence analysis of the virus in the original clinical sample of the same volunteer.

and from a placebo recipient (12), selected because of prolonged viral shedding (table 2). Virus was isolated from nasal washes and propagated once in MDCK cells before plaque assay. Isolates recovered from the drug recipient before and 2 days after the initiation of treatment were sensitive to the NA inhibitor in MDCK cells, whereas those recovered after 60 h were resistant (>50-fold increase in EC_{50} values for plaque size). The results demonstrated concordance between the emergence of a drug-resistance resistant phenotype, as assessed by plaque size and the acquisition of a mutation in the NA (274 His→Tyr; table 2 and figure 2), although these events were separated by 24 h (figure 1, *arrows*).

Plaque number reduction in MDCK cells. Drug-susceptibility phenotype was also tested by determining the reduction in number of virus plaques in the presence of oseltamivir. In contrast to results based on the plaque size reduction, resistant viruses ($EC_{50} > 3$ μM) were recovered commonly from both the drug and placebo recipients (tables 1 and 2). Isolates from these volunteers were initially susceptible to the drug, becoming resistant by 60 h of treatment. Importantly, all last-day isolates from the placebo, but not the drug recipients, were fully susceptible to the drug, on the basis of reduction of plaque size. Because the NA of those isolates remained sensitive to oseltamivir in the NI, changes in viral proteins other than the NA must have been responsible for the altered phenotype (NA-independent mechanism of resistance). One possibility is that a reduced affinity of the viral HA for cellular receptors of the MDCK cells facilitated the release of progeny viruses, thus reducing their susceptibility to NA inhibitors [3, 16]. To test

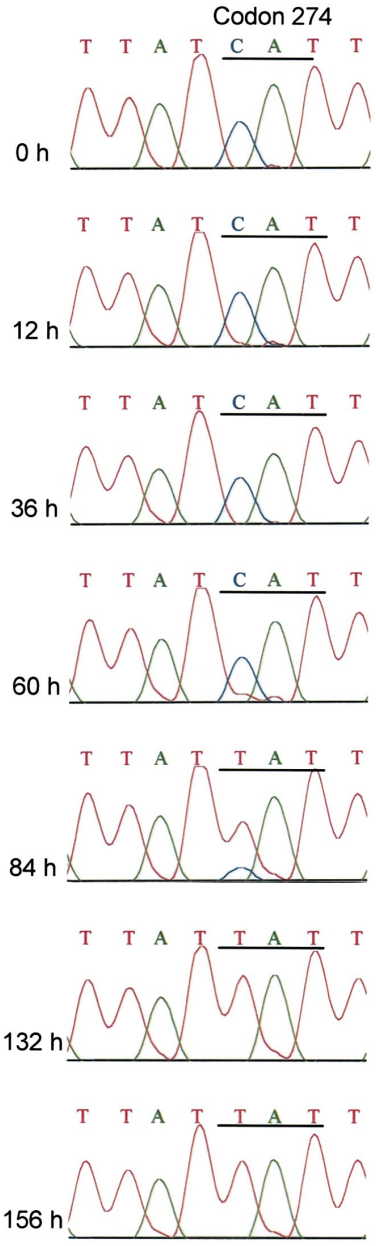

Figure 2. Sequence analysis of the neuraminidase (NA) gene of a virus from drug recipient 35. Total RNA was extracted from nasal washes collected before treatment (0 h) and at 12, 36, 60, 84, 132, and 156 h after treatment initiation. The NA gene was partially amplified by reverse transcriptase–polymerase chain reaction. Codon CAT encodes His, and codon TAT encodes Tyr.

this hypothesis, we compared the HA sequence and receptor-binding properties of the challenge virus with those of viruses isolated from the drug and placebo recipients.

Amino acid substitutions in the HA variants of the challenge virus. To identify substitutions in the HA that might be responsible for chicken egg adaptation (egglike HA variants), we generated individual clones of the challenge virus by limiting dilution in MDCK cells. For each clone, RT-PCR products encoding the HA1 region were obtained and sequenced. The predicted amino acid sequences then were compared, and residues characteristic of egg-adapted viruses of the H1N1 serotype were inspected [9, 13, 30]. This analysis revealed nucleotide variance at codon 225 in the receptor-binding site [31] and predicted that the challenge virus contained ⩾3 HA variants with Gly, Asn, or Asp at amino acid residue 225 (table 3). The Asp residue typically found in human viruses of H1N1 serotype was detected in 11% of the clones, whereas Gly and Asn, characteristic of egg-adapted human viruses (egglike HA variants), were identified in 58% and 32% of the clones, respectively (table 3). Although the clones harbored a few other nucleotide substitutions (data not shown), none was close to the HA receptor-binding region. Thus, the dominant HA variant in the challenge virus population had a Gly residue at position 225 (egglike HA variant).

HA variants recovered from placebo recipients. The HA sequences of virus isolates recovered from placebo recipients on the last day of viral shedding were also analyzed. Despite use of the same virus preparation to inoculate all volunteers, sequences of the recovered viruses varied widely (table 3). The predominant egglike HA variant of the challenge virus (Gly residue at 225) was recovered from only 25% of the placebo group. Another 25% shed the egglike HA variant with the amino acid substitution Asn at 225. Remarkably, half the placebo group shed the "non-egglike" HA variant with an Asp at 225 residue, which accounted for only a minor fraction of the challenge virus HA variants (table 3). Importantly, an amino acid residue not found in the challenge virus (Ala at 137) was identified in 42% of the isolates. The residues Ala 137 and Asp 225 are characteristic of human influenza H1N1 viruses and were the most common finding in the original clinical specimens before propagation in chicken eggs [30]. Of note, all GenBank sequences of human influenza viruses of H1N1 subtype contained Ala at 137. These findings indicate that Thr at 137 in the challenge virus is probably an egg-selected amino acid substitution, although it was detected infrequently in the HAs of viruses from clinical specimens [32].

Thus, most of the viruses recovered from placebo recipients on the last day of shedding had Asp225 with either Ala137 or Thr137 in the HA, a characteristic finding in human influenza viruses. For the sake of brevity, we will refer to virus isolate characteristics as either egglike or non-egglike, solely on the basis of HA sequence.

Because the HA variant Ala137/Asp225 was not detected in the challenge virus, we wished to learn when this variant had emerged. Thus, RNA was extracted from nasal washes collected from the placebo recipients on sequential days, followed by RT-PCR amplification and sequencing of the HA region encoding the receptor-binding site. By comparison with the challenge virus, the HA of isolates from a representative placebo recipient

Table 1. Drug susceptibility of the last-day isolates from experimentally infected subjects shedding influenza A virus for >3 days by neuraminidinase (NA) inhibition assay and plaque reduction in Madin-Darby canine kidney (MDCK) cells.

Subject	Treatment group	Day of isolation (postchallenge)	NA inhibition	Plaque reduction in MDCK cells		HA sequence	Receptor preference[a]
				Size	Number		
Challenge virus	—	—	S	S	S	Thr137/Gly225[b]	Neu5Ac(α2-3)Gal
18	Placebo	6	S	S	S	Thr137/Gly225	Neu5Ac(α2-3)Gal
25	Oseltamivir	7	S	S	S	Thr137/Gly225	Neu5Ac(α2-3)Gal
26	Oseltamivir	5	S	S	S	Thr137/Gly225	Neu5Ac(α2-3)Gal
39	Oseltamivir	6	S	S	S	Thr137/Gly225	Neu5Ac(α2-3)Gal
2	Oseltamivir	5	S	S	R	Ala137/Asp225	Neu5Ac(α2-6)Gal
9	Oseltamivir	7	S	S	R	Ala137/Asp225	Neu5Ac(α2-6)Gal
12	Placebo	5	S	S	R	Ala137/Asp225	Neu5Ac(α2-6)Gal
20	Oseltamivir	6	S	S	R	Ala137/Asp225	Neu5Ac(α2-6)Gal
48	Placebo	7	S	S	R	Ala137/Asp225	Neu5Ac(α2-6)Gal
52	Placebo	7	S	S	R	Ala137/Asp225	Neu5Ac(α2-6)Gal
71	Placebo	6	S	S	R	Ala137/Asp225	Neu5Ac(α2-6)Gal
77	Placebo	6	S	S	R	Ala137/Asp225	Neu5Ac(α2-6)Gal
5	Oseltamivir	7	R	R	R	Thr137/Gly225	Neu5Ac(α2-3)Gal
35	Oseltamivir	>8	R	R	R	Thr137/Asn225	Neu5Ac(α2-3)Gal

NOTE. Viruses were considered to be resistant in the plaque assay if their EC_{50} values were increased >50-fold relative to the challenge virus ($EC_{50} < 0.03\ \mu M$, for both plaque size and number; see also table 2). Enzyme resistance was defined as a >1500-fold increase in the IC_{50} values due to a His→Tyr substitution at residue 274 of the NA. HA, hemagglutinin; R, resistant; S, sensitive.

[a] Receptor preference was based on results of an HA assay, as described in Methods.

[b] Sequence of the dominant HA variant (see also table 3).

(52) showed 2 different changes at codon 225; however, the only sequence detected from 36–84-h was Asp (GAT; figure 3, *right*). The HA variant with Thr at residue 137 was replaced by a variant that carried Ala (figure 3, *left*). Hence, replication of the egg-adapted challenge virus in placebo recipients led to the emergence of variants in which the HA lacked egglike amino acid substitutions at residues 137 and 225.

HA variants recovered from oseltamivir recipients. Viruses recovered on the last day of shedding were divided into 2 groups: those from volunteers given low drug doses (20 mg twice daily) and from those given high doses (100 mg twice daily or 200 twice or once daily). By sequence analysis, the frequency of recovery of non-egglike HA variants (Ala137/Asp225 and Thr137/ Asp225) was significantly lower in the high-dose drug recipients than in the placebo group: 10% (4/40) versus 50% (6/12; $P < .01$, Fisher's exact test). Such variants were recovered at an intermediate frequency, 31%, in the low-dose drug group (table 3). The egglike HA variants Thr137/Gly225 and Thr137/Asn225 were equally represented among viruses recovered from volunteers receiving high doses of oseltamivir (43% and 42%, respectively). Finally, a minor HA variant (5% recovery frequency) was characterized by Lys137, another common egglike substitution [33]. These findings clearly indicate the dominance of egglike HA variants among last-day isolates from high-dose oseltamivir, compared with placebo recipients.

Receptor-binding properties of HA variants. A modified HA assay was used to compare receptor-binding properties of the challenge virus and its HA variants [22]. Although the HA variant Thr137/Gly225 efficiently agglutinated erythrocytes with predominantly α2-3-linked receptors (32 hemagglutinating units [HAU]), its agglutination of erythrocytes carrying receptors with predominantly α2-6-linkages was substantially impaired at 37°C (4 HAU). The opposite was observed for the HA variant Ala137/Asp225 (2 HAU vs. 16 HAU). Two other HA variants, Thr137/Asn225 and Thr137/Asp225, produced an

Table 2. Acquisition of drug resistance by isolates collected sequentially from subjects treated with oseltamivir or placebo.

Subject, time after treatment, h	$EC_{50}(\mu M)$		Drug susceptibility phenotype in MDCK cells
	Plaque size	Plaque number	
Challenge virus			
—	<0.03	<0.03	S/S
Drug recipient 35			
0	<0.03	0.03	S/S
24	<0.03	0.06	S/S
36	<0.03	0.06	S/S
60	1.6	>3	R/R
84	1.8	>3	R/R
108	5.8	>3	R/R
132	1.6	>3	R/R
156	1.9	>3	R/R
Placebo recipient 12			
0	<0.03	0.12	S/S
24	<0.03	0.06	S/S
36	<0.03	0.1	S/S
60	<0.03	>3	S/R
84	Negative[a]	Negative	Negative

NOTE. MDCK, Madin-Darby canine kidney; R, resistant phenotype (EC_{50} value increased >50-fold); S, sensitive ($EC_{50} < 0.03\ \mu M$).

[a] Virus was not recovered.

Table 3. Frequency of isolation of variants with amino acid substitutions in the receptor-binding site of hemagglutinin (HA).

		Frequency of isolation, %				
		Egglike			Non-egglike	
Treatment	No. of isolates	Thr137 Gly225	Thr137 Asn225	Lys137 Asp225	Thr137 Asp225	Ala137 Asp225
Challenge virus, clones[a]	19	58	32	0	11	0
Placebo	12	25	25	0	8	42
Oseltamivir, 20 mg	13	15	54	0	0	31
Oseltamivir, 100–200mg	40	43	42	5	5	5

[a] Challenge virus was cloned in Madin-Darby canine kidney cells by limiting dilution, and the HAs of individual clones were sequenced.

intermediate agglutination result in HA assay (not shown), both displaying a higher affinity for α2-3-linked receptors than did the Ala137/Asp225 variant. The HA variant Lys137/Asp225 effectively agglutinated erythrocytes of both types at both 4°C and 37°C (32 HAU). Thus, in terms of receptor specificity, the HA variant Ala137/Asp225, recovered from 42% of placebo recipients but only 5% of drug recipients, was the most closely aligned with human influenza viruses [13, 30, 32]. By contrast, the codominant HA variants of the challenge virus displayed receptor-binding properties more typical of egglike HA variants of human viruses.

Susceptibility of receptor-binding variants to oseltamivir in humans. The possibility of a growth advantage of the non-egglike HA variants (Ala137/Asp225 and Thr137/Asp225) over the egglike variants cannot be addressed in challenge experiments with humans. We therefore sought to correlate the HA sequences of the last-day isolates with such parameters as the duration of viral shedding and virus titers by using the Mann-Whitney *U* test to compare differences between egglike and non-egglike viruses (table 4). The median durations of viral shedding among placebo recipients with non-egglike versus egglike HA variants were 5.0 and 4.0 days, respectively ($P = .06$).

The median duration of shedding by subjects with non-egglike HA variants was significantly longer in placebo recipients (5.0 days) than in the oseltamivir group (1.3 days; $P < .01$) and the load of virus was higher for placebo recipients with AUC values of 2.0 versus 0.4 ($P < .01$).

The replication of egglike HA variants tended to be 1.5 days shorter (4.0 vs. 2.5 days) in drug versus placebo recipients ($P = .06$). Among subjects receiving high-dose oseltamivir and shedding non-egglike HA variants, the reduction of shedding duration and virus titer AUC values were about half the corresponding values for the egglike HA variants (table 4). Despite the limitation related to the small sample sizes, these results suggest that the inhibitory effects of oseltamivir were more pronounced in volunteers who shed non-egglike HA variants. Both viruses that acquired the drug-resistant NA were the egglike HA variants.

Discussion

The goal of this study was to assess the emergence of drug-resistant variants in volunteers experimentally infected with the H1N1 strain of influenza virus and treated with the NA inhibitor oseltamivir. Our findings indicate that oseltamivir treatment can select for a NA active-site mutation that confers enzyme drug resistance in a minority of study participants. Changes in the HA were also commonly found, even in the absence of selective drug pressure. We attribute the selection of HA variants to the adaptation of egglike virus for replication in the human respiratory tract. Our results suggest that such variants are more susceptible than the egglike HA variants to the NA inhibitory effects of oseltamivir. They also emphasize the methodological difficulties of monitoring for influenza virus resistance related to the mandatory use of egg-adapted chal-

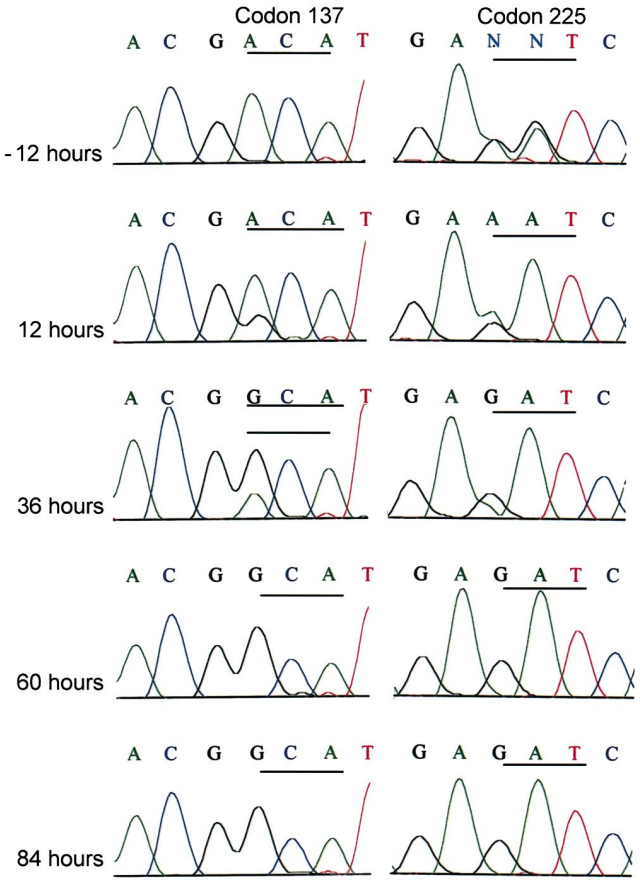

Figure 3. Sequence analysis of the hemagglutinin (HA) gene of a virus from placebo recipient 52. A partial HA sequence, including codons 137 *(left)* and 225 *(right),* is shown. Virus inoculation preceded treatment by 28 h; nasal washes were collected 12 h before treatment and at 12, 36, 60, and 84 h posttreatment. Codon ACA encodes Thr, GCA encodes Ala, GGT encodes Gly, AAT encodes Asn, and GAT encodes Asp.

Table 4. Relation of hemagglutinin (HA) variant selection to treatment status and viral shedding patterns in oseltamivir and placebo recipients.

HA variant type and treatment *(n)*	Median (range) days of virus shedding	Median (range) virus titers (AUC)
Non-egglike[a]		
Placebo (6)	5.0 (3.5–7.0)	2.0 (1.9–2.5)
Oseltamivir (4)[b]	1.3 (1.0–2.5)	0.4 (0.1–1.2)
Egglike		
Placebo (6)	4.0 (1.5–4.5)	1.2 (0.1–2.7)
Oseltamivir (34)[b]	2.5 (<1.0–5.5)	0.8 (0.1–2.9)

NOTE. AUC, area under the curve.

[a] Based on the HA sequence of last-day isolates. Compared with placebo recipients, the oseltamivir recipients shed viruses with non-egglike HA less often ($P < .01$).

[b] Drug doses, 100–200 mg twice daily and 200 mg once daily; the 2 isolates with resistant neuraminidinases were excluded.

lenge virus in human experiments and to the lack of a reliable cell culture assay.

Influenza viruses replicating in humans preferentially bind to the Neu5Ac(α2-6)Gal-terminated receptors of human respiratory epithelium [8, 9] and show reduced affinity for the Neu5Ac(α2-3)Gal-terminated receptors of target cells on chicken egg allantoic membranes [14]. Consequently, HA variants with increased affinity for allantoic membrane receptors outgrow the original human virus during passage in chicken eggs. They often have reduced affinity for α2-6-linked receptor determinants on human respiratory epithelium [9, 13]. It is not surprising, therefore, that the egg-adapted challenge virus used in our study generated a heterogeneous population of the HA variants (table 3). Two codominant variants possessed egglike mutations at position 225 (Gly or Asn) and displayed a typical egglike receptor-binding pattern. Particularly noteworthy was the emergence of HA variants with non-egglike sequences (Ala137/Asp225 and Thr137/Asp225) and with increased affinity for human receptors in half the placebo recipients. Our observations indicate that the egglike HA variants replicated less efficiently in humans than did the non-egglike HA variants. It is unclear why such selection was limited to only half the placebo recipients. One possibility is that the virus was cleared by immune mechanisms in some hosts before "fitter" variants could emerge and outgrow the challenge virus. Indeed, variants with increased affinity for human receptors tended to be recovered from subjects who shed virus for longer times.

Our results conflict with 2 previously published studies of egg-adapted influenza B viral replication in humans. In one, the HA of a vaccine strain retained its egglike characteristics after replication in 2 subjects [34], whereas, in the other, the HA of an egg-grown cold-adapted strain remained genetically stable during replication in 5 children [35]. Thus, the selective pressure favoring replication of "human-type" HA variants in human hosts may not be strong enough to allow consistently the emergence of such variants. It is also possible that our results reflect a more heterogeneous virus preparation or, perhaps, some distinctive property of the H1N1 virus.

Because of protocol constraints, we could not compare the drug susceptibility of egglike viruses versus non-egglike ones directly in humans. Nonetheless, it was possible to correlate the HA sequences of last-day isolates with the duration of viral shedding and with virus load, as estimated from AUCs for virus titers in sequentially obtained nasal washes (table 4). Although non-egglike HA variants (Ala137/Asp225 and Thr137/Asp225) replicated more efficiently in humans, the frequency of their isolation and their virus titers were significantly reduced in drug-treated subjects, compared with placebo recipients. These findings strongly suggest that either non-egglike variants are more sensitive to NA inhibitors or that reduction in viral replication associated with oseltamivir administration constrained the emergence of such mutants.

Our results also suggest that adaptation to receptors present on the human respiratory tract epithelium was responsible for the emergence of non-egglike HA variants in placebo recipients. Unlike the challenge virus, these variants preferably agglutinated erythrocytes carrying Neu5Ac(α2-6)Gal receptors. This preferential binding to receptors typical for human respiratory epithelium would be expected to increase the dependence of the variants on NA function in humans, hence explaining their greater susceptibility to the NA inhibitor. In an earlier study [22], we demonstrated that an influenza B mutant, isolated from an immunocompromised patient treated with zanamivir, another NA inhibitor, had a lower affinity for Neu5Ac(α2-6)Gal receptors than did the virus isolated before treatment. The defining mutation in the treatment-selected mutant was situated in the HA receptor-binding site and was characteristic of egg-adapted influenza B viruses. We conclude that affinity of the HA for Neu5Ac(α2-6)Gal receptors affects inhibition of viral replication by oseltamivir in humans.

Drug-sensitivity testing of HA variants in MDCK cells yielded anomalous results (table 1), particularly regarding the emergence of resistance in the placebo group (HA variants that were more sensitive to oseltamivir in humans appeared to be resistant in the MDCK cell plaque assay). Together, the data substantiate previous concerns that MDCK cells do not provide a reliable cell system for testing resistance to NA inhibitors [22, 26]. One of the reasons is that MDCK cells express receptors of both types and that the α2-3-linkages are more prevalent [14]. In the present study, the HA variant Ala137/Asp225, which binds poorly to Neu5Ac(α2-3)Gal receptors, was resistant to oseltamivir in MDCK cell assays, on the basis of EC_{50} values for plaque number. By contrast, the HA variants with a binding preference for Neu5Ac(α2-3)Gal receptors, including the challenge virus, were sensitive to the NA inhibitor in MDCK cells, except 2 viruses with resistant NA. Thus, in MDCK cells, the sensitivity of viruses to NA inhibitors greatly depends on HA affinity for Neu5Ac(α2-3)Gal receptors, which are predominant in these cells, whereas in humans, the affinity for abundant

Neu5Ac(α2-6)Gal receptors is critical. Our findings agree with previously published studies on resistance to NA inhibitors [4, 22, 36] and confirm that NI assays, supported by HA sequencing, should be used for monitoring the in-vivo susceptibility of virus isolates to this new class of antiviral drugs.

Both viruses with NA resistance to oseltamivir had a low affinity for Neu5Ac(α2-6)Gal receptors, because of a substitution at position 225 (Gly or Asn). This property, however, was acquired through egg adaptation of the challenge virus rather than through treatment pressure. The mutation in the NA active site (274 His→Tyr) was detected after 84 h of treatment by sequence analysis of viruses contained in the nasal wash (figures 1 and 2). We did not detect this mutation directly in viruses contained in the 60-h nasal washes (consensus sequence), but, when propagated twice in MDCK cells, the isolates demonstrated resistance in the plaque assay (MDCK cells) and had a His→Tyr substitution. Thus, in agreement with our previous observation [22], the propagation of virus specimens in MDCK cells encouraged the overgrowth of a NA mutant. The mechanism of such selection is presently unclear but could be related to a necessity to balance the HA and NA activities in a new host cell system [4, 37, 38].

Overall, the frequency of NA resistance in experimentally infected volunteers was low (4%) and arose in subjects treated with high doses of oseltamivir. The mutation His→Tyr at residue 274 of the NA is novel and was not recognized in adults receiving oseltamivir treatment for acute influenza A (H3N2) or B illness. In previous studies of a recombinant NA (N2 subtype), the replacement of His by Tyr at residue 274 caused a shift in the pH optimum of the enzyme (pH 6→5) with rapid loss of the mutant NA activity to only 10% of the wild-type enzyme activity at pH 8.0 [39]. When the resistant variant of A/Texas/36/91 (H1N1) carrying the His274Tyr mutation was tested in animal models, it demonstrated attenuated phenotype [23]. The observed attenuation of the mutant replication in mice and ferrets [17, 19, 23] may have been due to substantial reduction of NA activity. Such observations suggest that this mutant is at a significant disadvantage with regard to infectivity and transmissibility, compared with wild type. Further investigations are needed to elucidate a potential role of NA-independent mechanism(s) of virus resistance to NA inhibitors during acute influenza infection in humans.

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