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Oseltamivir Resistant Influenza A and B Viruses Pre- and Post Antiviral Therapy in Children and Young Adults with Cancer

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INTRODUCTION

Influenza A and B viruses are among the most common human respiratory viruses. In the US, influenza infection results in more than 225,000 hospital admissions and 36,000 deaths every year (1,2). Influenza causes significant morbidity and mortality in patients with compromised immune systems (3). Antiviral prophylaxis and chemotherapy is particularly important in immunocompromised individuals, such as those undergoing chemotherapy for cancer, because influenza vaccine is often poorly immunogenic and unlikely to be fully protective in these patients (4). In some immunocompromised individuals, however, drug-resistant influenza viruses can develop and reduce the efficacy of chemoprophylaxis and treatment.

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The neuraminidase inhibitors (NAIs) oseltamivir and zanamivir have been the cornerstone of anti-influenza therapy in the recent years. Decreased susceptibility to NAIs can occur as a result of mutations at the conserved residues in the active site of NA, which can limit the interaction of the drug with the sialic acid binding pocket of NA (NA-dependent resistance) (5). Previous studies have shown that resistance to NAIs varies with the NA subtype of influenza virus and that distinct NA mutations may cause different patterns of resistance (6). Four major mutations in NA that can cause NAI resistance have been reported. In influenza viruses of the N2 subtype, a glutamic acid to valine substitution in residue 119 (E119V, N2 numbering here and through the text) confers resistance to oseltamivir but not to zanamivir; an arginine to lysine substitution at position 292 (R292K) confers resistance to both NAIs. Most H1N1 viruses circulating since the 2007-2008 influenza season have a histidine to tyrosine substitution at residue 274 (H274Y), which is also associated with resistance to oseltamivir but not zanamivir (6,7). Other mutations such as an asparagine to serine substitution at position 294 (N294S), have been reported in both N2- and N1-containing viruses; these mutations are associated with a greater loss of *in vitro* susceptibility to oseltamivir in N2 than in N1 viruses but strains possessing these mutations retain susceptibility to zanamivir (8,9). E119V and N294S mutations occur in the framework region of the NA (10). Resistance to the second class of anti-influenza drugs, adamantanes, results in most cases from a single serine to asparagine amino acid replacement (S31N) in the matrix M2 protein, which can interfere with the drug's ability to block M2 ion channel activity and viral replication (11).

Immunosuppression enhances the opportunity for viral replication to occur for extended periods. This is a risk factor for the emergence of drug resistance and for prolonged viral shedding and transmission (7,12). Despite immunocompromised patients being at high risk of developing antiviral resistance, our understanding of the circumstances under which resistant viruses arise in this population and the clinical significance of resistance remains incomplete. We therefore studied immunocompromised children and young adults with cancer treated at St. Jude Children's Research Hospital (SJCRH) who developed influenza infection between 2002 and 2008 and were treated with oseltamivir.

MATERIALS AND METHODS

Study population

SJCRH provides comprehensive care for pediatric patients with cancer and other types of immunodeficiency. All clinical and laboratory information related to influenza and all other acute illnesses experienced by these patients is tracked. Children and young adults aged 0-25 years with cancer and culture-confirmed influenza infection who were treated with oseltamivir between January 1, 2002 and March 1, 2008 were identified by reviewing diagnostic microbiology laboratory results. Initial diagnostic tests for influenza and follow-up testing were performed at the discretion of treating oncologists. Demographic and clinical features were obtained by reviewing medical records. The SJCRH Institutional Review Board approved the study.

Definitions

Prolonged influenza infection and viral shedding were defined as symptomatic respiratory tract disease and viral shedding for ≥ 14 days after primary laboratory confirmation of infection (13-16). The last day of viral shedding was defined as the day of the first negative respiratory sample from a patient. Influenza was considered a nosocomial infection if the illness developed ≥ 4 days after hospitalization. Oseltamivir (30 mg for children weighing ≤ 15 kg, 45 mg for those weighing 15.1-23 kg, 60 mg for those weighing 23.1-40 kg and 75 mg for persons weighing >40 kg) was taken orally twice per day and amantadine (5 mg/kg/

day for children weighing <40 kg and 100 mg for persons weighing ≥40 kg) was taken orally twice daily.

Viruses and detection of anti-viral resistance

Viruses were isolated from clinical respiratory samples by passaging 1–2 times in Madin-Darby canine kidney (MDCK) cells, as previously described (17). Hemagglutinin (HA) subtypes were determined by HA inhibition assay (18).

Stock solutions of oseltamivir carboxylate (the active ingredient of oseltamivir) and zanamivir were provided by Hoffmann La Roche (Basel, Switzerland) and were dissolved in distilled water and aliquots stored at –20°C until used. NA enzyme inhibition was measured using a modified fluorescence-based assay as described previously (19), using 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA; Sigma, St. Louis, MO) at a final concentration of 100 μ M as a substrate. Assays were performed in triplicate. The IC₅₀ was defined as the concentration of NAI necessary to reduce NA activity by 50% relative to that of a reaction mixture containing virus but no inhibitor. Full-length NA and matrix (M2) genes of viral isolates found to be resistant to at least one neuraminidase inhibitor were sequenced as previously described (20). Nucleotide sequences of these genes have been deposited in the GenBank database under accession numbers CY068886 - CY068895. There is no generally accepted definition of the IC₅₀ values for NAI-resistant influenza viruses, but a change in IC₅₀ of ≥ 10-fold in a virus isolated from a patient before and after NAI treatment is commonly taken as the hallmark of resistance (21), and will be used to define resistance in this study.

Statistical analysis

The paired two-tailed *t*-test was used to compare the duration of the symptoms between patients who received 1 course of oseltamivir (twice daily for 5 days) and those who received more than 1 course of oseltamivir. Data were analyzed by using SAS software, version 9.1 (SAS Institute, Cary, NC).

RESULTS

Demographic characteristics

A total of 102 patients with cancer were diagnosed with influenza during the study period. Fifty-one patients had culture-confirmed influenza infection. The twelve patients who had viral isolates obtained both before and after oseltamivir therapy was initiated were selected for study. Of the 40 original isolates from these patients, 27 (12 before therapy and 15 after initiation of oseltamivir therapy) could be re-cultivated for analysis of resistance.

Supplemental Digital Content 1, <http://links.lww.com/INF/A646> (table) shows the demographic characteristics of the patients in the study. The mean age (10.5 years, range 1.1–23.0 years), gender distribution (male 58.3%), and underlying malignancy (hematological malignancy 10 patients (83%)) were not statistically different from those of the overall population of patients with cancer and influenza treated at SJCRH during the study period (data not shown). All patients were receiving immunosuppressive therapy. Among children with hematological malignancies, 9 had acute leukemia, including 2 in the induction phase of their chemotherapy, and 3 were in remission at the time of their influenza infection. Four patients received a HSCT (3 allogeneic) 3–104 days prior to the diagnosis of influenza infection.

Clinical characteristics

For the majority (10 of 12) patients, information about whether they had received the influenza vaccine in the current season was not available. Fever, cough and coryza were the most common signs and symptoms. Only 1 patient did not have prolonged respiratory symptoms. The mean duration of symptoms was comparable to those of the overall population of patients with cancer and influenza treated at SJCRH during the study period (mean 32 ± 35 days vs. $17 \text{ days} \pm 14$ days, $P=0.731$, data not shown). Eleven patients were hospitalized and 7 of these hospitalizations were solely attributable to influenza symptoms. The median duration of the hospitalization for patients hospitalized due to influenza symptoms was 7 days (range: 2-13 days).

The mean absolute neutrophil count (ANC) and absolute lymphocyte count (ALC) at the time of the onset of influenza symptoms were $957/\text{mm}^3$ (range 100-12,400) and $545/\text{mm}^3$ (range 192-3,534), respectively, and these were comparable to those of the overall population of patients with cancer and influenza treated at SJCRH during the study period (mean ANC 1629 ± 2759 , $P=0.455$; mean ALC 511 ± 886 , respectively, $P=0.901$; data not shown).

Serum concentrations of hepatic transaminases were available for 10 patients [median aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels: 30 U/L and 46 U/L respectively]. Values were above the upper limit of normal for age in 4 patients but only 2 were more than double this value. There were no other notable laboratory abnormalities.

Two infections (16.7%) were considered nosocomial. No deaths were attributed to influenza. Complications included respiratory failure ($n=1$), pneumonia ($n=2$), sinusitis ($n=4$) and acute otitis media ($n=1$). Therapy was delayed because of influenza infection in 6 of 8 patients receiving chemotherapy (median delay 10 days, range 6-21 days).

Serial viral isolates were obtained over ≥ 14 days from 8 patients; 8 of these patients shed virus for prolonged periods despite antiviral treatment.

Antiviral treatment

Of the 12 patients, 11 were treated with oseltamivir only and 1 patient was treated with oseltamivir for 1 day followed by amantadine for 5 days (during an influenza season when treatment with amantadine was recommended because of few circulating amantadine-resistant strains) (see Table, Supplemental Digital Content 1, <http://links.lww.com/INF/A646>) (9, 22). Antiviral therapy was started within 48 h of onset of symptoms in 9 patients. Of the 11 patients who received oseltamivir only, the duration of antiviral therapy was 5 days for 6 patients and >5 days for 5 patients. The duration of symptoms after commencing antiviral treatment was similar for patients receiving 5 days and those receiving more than 5 days of therapy (mean 33.0 ± 8.3 days vs. 26 ± 17.9 days, respectively, $P=0.444$).

Virological characteristics

Overall, 9 patients had influenza A [8 influenza A (H3N2), 1 influenza A (H1N1)] and 3 had influenza B virus infection (Table 1). A total of 27 isolates were studied, including those from 8 patients from whom 2 or more serial cultures had been obtained over the course of their infection; and 4 isolates obtained before initiation of therapy from 4 patients. Oseltamivir-resistant viruses were isolated from 4 of 12 (33%) patients [3 influenza A (H3N2) and 1 influenza B] (Table 1). Each was isolated during a different influenza season, during which between 7 and 23 additional patients had laboratory-confirmed influenza.

In two cases (patients 7 and 12), oseltamivir resistant virus was isolated from samples obtained prior to antiviral therapy (see Figure, Supplemental Digital Content 2, <http://links.lww.com/INF/A648>) (Table 1). An oseltamivir resistant virus was isolated from patient 2 on day 5 of oseltamivir therapy (the first specimen available from this patient) and again from a sample collected on day 3 of discontinuing treatment. In patient 6, resistance was first detected on day 7 of oseltamivir treatment in the third of 6 serial cultures obtained. All viruses were susceptible to zanamivir.

Sequence analysis of viral isolates

Mutations in the NA gene were identified in oseltamivir resistant influenza viruses obtained from 4 patients (see Table, Supplemental Digital Content 1, <http://links.lww.com/INF/A646>; Figure, Supplemental Digital Content 2, <http://links.lww.com/INF/A648>). An E119V substitution in the NA was detected in all three resistant influenza A (H3N2) strains obtained from patients 2, 6 and 7. For patient 7, oseltamivir resistance preceded the initiation of oseltamivir treatment. In patient 6, a viral isolate collected on day 7 of therapy revealed a mixed population of wild-type virus and E119V mutant quasi-species. The E119V mutant then emerged as the dominant strain in the next isolate collected on day 9 of therapy. For patient 12, influenza B virus harboring an N294S mutation in the NA was detected before therapy. Although isolates from patients 10 and 11 demonstrated higher IC₅₀ values than those published for oseltamivir-sensitive influenza B strains (23-25), sequence analysis of the NA gene of these viral isolates did not reveal any mutation in sites that had been reported to confer resistance.

Susceptibility to adamantanes was tested for all oseltamivir-resistant influenza A (H3N2) viruses by sequence analysis of the M gene; all strains had an S31N mutation in the transmembrane region of the M2 protein and were therefore resistant to these drugs (data not shown).

DISCUSSION

The frequency at which oseltamivir resistant influenza strains arise and are transmitted among immunocompromised individuals is unknown. During the first 3 years (1999-2001) after the introduction of NAIs, low rates of resistance (0.22% to 0.41%) were reported in circulating influenza A (H1N1) and A (H3N2) viruses (26). Levels of resistance remained low until 2007, after which widespread resistance of influenza A (H1N1) viruses to oseltamivir was reported worldwide (27). At present, most influenza A (H3N2) strains and pandemic 2009 H1N1 viruses isolated in the US remain susceptible to oseltamivir (28).

There have been few reports of drug resistant influenza strains in immunocompromised patients (12,17,29-32). Considering the low proportion of NAI resistance (4% for children aged 1-12 years and 0.4-1% for adults) observed in controlled clinical trials on NAIs (14,33,34), as well as the paucity of reports on the transmission of resistant strains or the documentation of primary (*de novo*) resistance to NAIs (25,35), the extent of oseltamivir resistance noted in the patients in our study (33% of patients at some point during their infection) was unexpected. The demographic and clinical characteristics of the 12 patients and their influenza isolates included in the study were comparable to the overall population of patients with cancer and influenza treated at SJCRH during the study period. It is possible that those patients who had serial cultures performed were subtly different from the overall population; however, the duration of the influenza symptoms in this cohort was not statistically different from that of the overall population. In addition, half of the resistant viruses in our study were detected early in the course of patients' illnesses therefore the selection of these isolates could not be influenced by the clinical course of infection. Furthermore, not all viruses were tested after oseltamivir treatment and some resistant

strains may not have been detected. Our cross-sectional analysis of 12 immunocompromised patients with cancer, therefore, indicates that the proportion of resistant influenza viruses among this population may be significant.

In our study, 1 influenza A (H3N2) (patient 7) and 1 influenza B (patient 12) strain were resistant to oseltamivir, despite neither patient having received oseltamivir therapy. The influenza A (H3N2) virus harbored an E119V NA mutation, which has been reported in oseltamivir-treated patients (31,36), but this is the first report of this mutation arising prior to oseltamivir exposure. The resistant influenza B harbored an N294S NA mutation. This mutation has been described in N2 and N1 subtypes (8) as well as in 2 fatal cases of avian influenza A (H5N1) viruses and confers a ≥ 15 -fold reduction in oseltamivir susceptibility (37). This is the first report of this mutation occurring in an influenza B virus. It is possible that resistance arose spontaneously in these two cases. However, the most likely scenario is that this virus was transmitted from another person with influenza who had been treated with oseltamivir - no information about ill contacts, however, was available.

Surveillance studies on NAI sensitive influenza viruses reported that oseltamivir IC₅₀ values for influenza B viruses are commonly ~10-fold higher than for influenza A viruses, and depending on the assay method used, may be >70-fold higher (23,38,39). This may explain the low sensitivity of influenza B viruses to oseltamivir in the clinical setting (40). Oseltamivir IC₅₀ values for the two oseltamivir sensitive influenza B viruses (patients 10 and 11) in our study were similar to IC₅₀ values previously reported for oseltamivir susceptible influenza B viruses (23,25,39,41,42) and were higher compared to the IC₅₀ values for sensitive influenza A viruses.

Additional amino acid substitutions in the NA (I194V, K199E, and L372S) were detected in 1 patient with influenza A (H3N2) virus (patient 2). These NA mutations have been reported in isolates obtained from other immunocompromised patients, but their contribution to drug resistance still uncertain at present (31).

In patient 6 a mixture of oseltamivir-sensitive and -resistant influenza A (H3N2) virus quasi-species was detected on day 7 of therapy. Subsequently a homogeneous population of resistant virus was detected on day 9 of treatment (5). It is possible that interruption in the administration of oseltamivir therapy early in this patient's treatment resulted in suboptimal plasma drug concentrations; permitting the escape of resistant quasi-species and subsequent development of resistant viruses. However, resistant viral quasi-species have been reported to emerge early in the treatment of immunocompromised patients despite good adherence to antiviral therapy (12) and the role of adherence to antiviral therapy in preventing the emergence of quasi-species and resistant virus remains to be clarified.

This possibility of primary or the rapid emergence of antiviral resistance should be considered when planning therapy for immunocompromised patients with influenza infection. Like the majority of influenza A (H3N2) viruses circulating globally (9), all 3 oseltamivir-resistant influenza A (H3N2) viruses detected in this study possessed a mutation of the M2 protein that confers amantadine resistance and, therefore, were susceptible to only one FDA-approved anti-influenza drug (zanamivir). It is common for physicians to extend the course of oseltamivir treatment if patients do not show improvement but our study illustrates that this may not be appropriate. Antiviral susceptibility testing is not routinely performed in most clinical settings but this study suggests that identification of antiviral-resistant viruses can be especially helpful in managing immunocompromised patients.

As in previous studies (12,29,31), influenza infection in our patients was characterized by prolonged (>2 weeks) respiratory symptoms. Immunocompromised patients may also shed influenza for prolonged periods, often despite administration of antiviral therapy, increasing

the chances for emergence of resistance and increased transmissibility of the virus (30,31). Prolonged viral replication (≥ 14 days) was documented in 8 of 12 patients for whom serial samples were obtained over the course of at least 14 days; although limited, these data suggests that prolonged shedding may be common in immunocompromised individuals. These findings are of particular concern in the health care setting where prolonged isolation precautions are important to prevent nosocomial outbreaks of resistant influenza viruses.

Our study has some limitations that must to be addressed. Specimens were not collected systematically before and after antiviral therapy and technical problems prevented the study of all viruses. This study suggests, however, that oseltamivir-resistant influenza may be more common among immunocompromised patients than previously believed, even when resistant strains are uncommon in the community. Serial cultures and determination of antiviral susceptibilities are required to optimally manage immunocompromised patients with influenza and precautions to limit viral transmission to others may need to be greatly prolonged. Future research should be focused on the complete characterization of the incidence and clinical impact of resistant viruses in immunocompromised individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TABLE 1

Susceptibility to Oseltamivir of Influenza A and B Virus Isolates.

Patient	Isolation year	Influenza subtype	Day of sample collection [*]	Susceptibility to oseltamivir (mean IC ₅₀ ± SD, nM) [‡]	NA mutations
1	2008	A (H3N2)	-1	0.41 ± 0.11	NT
			16	0.83 ± 0.97	NT
2	2002	A (H3N2)	5	137.25 ± 7.14	E119V
			8	134.37 ± 29.41	E119V
			-1	0.67 ± 0.42	NT
3	2004	A (H3N2)	25	0.39 ± 0.22	NT
			39	0.85 ± 0.23	NT
			97	0.83 ± 0.19	NT
4	2008	A (H3N2)	0	0.54 ± 0.45	NT
5	2002	A (H3N2)	0	0.32 ± 0.10	NT
			6	0.25 ± 0.08	NT
			2	0.31 ± 0.16	‡
6	2008	A (H3N2)	4	0.30 ± 0.05	E119G/E119V [§]
			7	180.26 ± 76.48	E119V
7	2005	A (H3N2)	-1	183.35 ± 9.73	E119V
			-1	2.99 ± 0.63	NT
8	2008	A (H3N2)	15	0.45 ± 0.24	NT
			26	0.75 ± 0.41	NT
9	2008	A (H1N1)	-1	0.98 ± 0.37	NT
10	2005	B	-3	12.00 ± 18.04	NT
			-2	42.27 ± 15.62	NT
11	2003	B	5	31.68 ± 20.00	NT
			0	731.86 ± 155.12	N294S
12	2003	B	25	772.75 ± 103.57	N294S

* With respect to the first day of antiviral therapy.

[¶] All influenza viruses were susceptible to zanamivir (mean IC₅₀ values range from 0.01 to 0.84, nM).

[‡] No mutations were detected.

[§] Mixed quasi-species. Amino acid numbering is based on N2 NA.

SD indicates standard deviation; NT indicates not tested.