Prolonged Influenza Virus Infection during Lymphocytopenia and Frequent Detection of Drug-Resistant Viruses

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The factors that cause prolonged human influenza virus respiratory tract infection and determine its clinical impact and the development of drug-resistant viruses are unclear. During a 3-year period, symptomatic influenza virus excretion for ⩾2 weeks was observed among 8 immunocompromised patients and found to be associated with lymphocytopenia at onset (8 of 8 patients) more often than with granulocytopenia (2 of 8 patients) or monocytopenia (2 of 8 patients). Six (75%) of 8 patients developed influenza lower respiratory tract infection (10 episodes), and receipt of oseltamivir treatment was significantly associated with clinical improvement (8 of 8 episodes vs. 0 of 2 untreated episodes; $P = .02$). Complete viral clearance was strongly correlated with lymphocyte reconstitution ($P = .04$) but was never observed during the first 2 weeks after oseltamivir treatment. Neuraminidase inhibitor–resistant influenza viruses emerged in 2 (67%) of 3 patients eligible for resistance analysis. In conclusion, prolonged influenza virus infection was associated with lymphocytopenia, influenza lower respiratory tract infection, and frequent development of drug resistance during antiviral therapy. Clinical improvement in influenza lower respiratory tract infection is observed during oseltamivir treatment, but complete viral clearance is dependent on lymphocyte reconstitution, irrespective of receipt of antiviral medication.

Human influenza viruses cause frequent morbidity and mortality, particularly in high-risk populations [1]. Antiviral drugs are important for treatment of infection and control of transmission during seasonal and pandemic influenza [2]. The recent and unexplained widespread emergence of circulating human influenza viruses with reduced sensitivity to adamantanes and neuraminidase (NA) inhibitors (NAIs) is therefore of major concern [2–5]. The potential modes by which NAI-resistant influenza viruses emerge merit further analysis. Particularly among children and immunocompromised hosts, prolonged viral excretion is associated with a higher incidence of drug resistance during antiviral therapy [6–13]. The frequency with which such prolonged influenza viral excretion occurs, the clinical consequences of this condition, the development of antiviral resistance, and its correlation with impaired cell-mediated immunity are unclear, however [1, 14]. During a 3-year period, these questions were addressed through careful analysis of findings in 8 patients who presented with prolonged influenza virus respiratory tract infection.

METHODS

Patients and specimens. The study included patients with prolonged influenza virus respiratory tract infection (defined below) observed at Leiden University Medical Center from March 2005 through April 2008. Respiratory specimens were obtained during episodes of symptomatic respiratory tract disease. Routine follow-up sampling was not performed during asymptomatic episodes that occurred in patients with established prolonged influenza virus infection, except for patient 1 and on single occasions for patients 3–6 to document complete viral clearance. Influenza virus type A or B RNA was detected in respiratory specimens by reverse-transcriptase polymerase chain reac-

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tion (PCR) [15]. Medical records were reviewed for underlying immunodeficiency and clinical characteristics during presentation, follow-up, and outcome, including upper or lower respiratory tract infection symptoms, intubation, antiviral therapy, viral clearance, leukocyte counts, hematopoietic stem cell transplantation (HSCT), and survival. Minimum cell count percentages for granulocytes, monocytes, and lymphocytes determined within 1 week before or after initial detection of viral RNA (i.e., onset) were correlated with percentages determined within 2 weeks after final influenza virus RNA detection or within 1 week before fatal influenza pneumonia.

Definitions. Prolonged influenza virus respiratory tract infection was defined as PCR-positive status combined with symptomatic respiratory tract disease for ⩾2 weeks after primary laboratory confirmation of influenza virus infection. This definition is based on documented maximum durations of excretion and approximate median duration of symptoms among immunocompetent children (duration of excretion, ⩽14 days; duration of symptoms, 7.1 days) [16, 17] and adults (duration of excretion, ⩽5.5 days; duration of symptoms, 6 days) [18, 19] and mean durations of excretion among immunocompromised autologous HSCT recipients (6.7 days) and allogeneic HSCT recipients (11.1 days) [20]. Influenza lower respiratory tract infection was diagnosed clinically (as dyspnea) and by PCR, and fatal influenza pneumonia was diagnosed by histology and PCR of pulmonary tissue specimens.

Minimum reference cell counts for granulocytes (1800 cells/mm^3), monocytes (135 cells/mm^3), and lymphocytes (900 cells/mm^3) were used for adults, and pediatric values were age dependent. These thresholds were used to determine granulocytopenia, monocytopenia, and lymphocytopenia.

Precursor virus was defined as influenza virus detected in a patient before antiviral therapy was started. Seasonal viruses were defined as community-acquired influenza viruses collected from patients with influenzalike illness or acute respiratory infection who had consulted a general practitioner involved in the sentinel surveillance system in The Netherlands. Baseline NAI susceptibility for seasonal viruses was defined as the IC_{50} value (i.e., the mean concentration of NAI needed to inhibit the NA enzyme activity by 50%), after removal of outlier IC_{50} values. NAI drug resistance was defined as a viral IC_{50} value ⩾8-fold higher than that of the corresponding sensitive precursor virus [21] and detection of specific NA gene mutations known to confer NAI drug resistance. In the absence of a precursor virus isolate, the IC_{50} of a presumed drug-resistant isolate was compared with baseline NAI susceptibility to calculate the *n*-fold change in drug susceptibility.

Influenza virus characterization and drug susceptibility testing. Influenza viruses were cultured in Madin-Darby canine kidney cells from specimens stored at −80°C. Antigens were characterized by hemagglutination inhibition testing, as described elsewhere [22]. Phenotypic susceptibility (IC_{50}) for oseltamivir carboxylate (GS4071; Roche Diagnostics) and zanamivir (GG167; GlaxoSmithKline) was determined as described elsewhere [23]. Emergence of NAI-resistant viruses was confirmed by phenotypic and sequence analysis of precursor viruses.

Influenza virus RNA was extracted from culture supernatant or clinical specimens using the QIAamp Viral RNA kit (Qiagen). Specific primers (available on request) were used for transcription (ThermoScript reverse transcriptase; Invitrogen) and cDNA amplification (Phusion High-Fidelity Taq polymerase; Finnzymes) of influenza NA, hemagglutinin (HA) and matrix 2 (M2) genes. DNA sequences were analyzed using Bionumerics software (version 5.1; Applied Maths).

Data analysis. Patients treated with oseltamivir and untreated patients were compared with respect to clinical recovery from lower respiratory tract infection, by use of Pearson's χ^2 tests and 2-sided Fisher's exact tests (SPSS software, version 14.0.2; SPSS). The percentages of the granulocyte, monocyte, and lymphocyte minimum cell counts at onset were compared with the percentages of these counts during viral clearance, by use of the Wilcoxon matched-pairs signed rank test.

RESULTS

Patients with prolonged influenza virus respiratory tract infection. Eight immunocompromised patients (median age, 52 years [range, 0–66 years]) were identified who had prolonged influenza virus excretion (median duration of excretion, 29.5 days [range, 14–275 days]) and symptomatic respiratory tract disease (table 1). These patients' underlying diseases included hematological malignancy ($n = 7$) and severe combined immunodeficiency ($n = 1$). The duration of symptoms observed by healthcare workers before the first detection of influenza virus was 2 days for patient 5 (coryza and fever), 2 days for patient 8 (dyspnea), 4 days for patients 2 and 3 (dyspnea and fever), 14 days for patient 6 (cough), and 20 days for patient 7 (cough). For patient 1, the duration of symptoms (cough) was 6 weeks before the first viral isolation, according to the history provided by a parent. Symptom duration could not reliably be determined for patient 4, who had chronic underlying pulmonary disease. Lymphocytopenia was more common at the onset of prolonged influenza virus infection (8 of 8 patients) than were granulocytopenia (2 of 8 patients) or monocytopenia (2 of 8 patients).

Influenza virus characterization. Viruses cultured from 4 patients were characterized as influenza A/Wyoming/003/03 (H3N2)–like (patient 1), A/California/007/04 (H3N2)–like (patient 2), A/Wisconsin/67/05 (H3N2)–like (patient 5) matching corresponding vaccine strains, and A/Solomon Islands/03/06 (H1N1)–like with a poor vaccine match (patient 8) (i.e., ~16-fold difference by duplicate hemagglutination inhibition titers). The remaining 4 influenza viruses A (H3N2) (patient 3), A (H1N1) (patient 6), and B (patients 4 and 7) could not be cultured or remained uncharacterized.

Table 1. Patients with prolonged influenza virus excretion and sequential susceptibility results.

Patient number	Patient sex	Patient age, years	Underlying disease (duration)	Flu typing (duration of excretion)[a]	Infection date	Antiviral therapy	Relevant genetic amino acid substitutions (GenBank accession no.)			NA inhibition assay, IC_{50}, nmol/L	
							NA gene[b]	HA1 gene[c]	M2 gene[d]	Oseltamivir	Zanamivir
8	M	66	Acute myelogenous leukemia (46 months), allogeneic stem cell transplantation (34 months), donor lymphocyte infusions (25 months)	A H1N1 (23 days)	Day 1	None	H274Y (AB462370)	NP	NP	471	0.96
7	F	58	Acute myelogenous leukemia (5 months), allogeneic stem cell transplantation (3 months)	B (14 days)	Day 0	None	None (AB462368)	NP	. . .	9.9	4.4
6	M	60	Non-Hodgkin lymphoma (7 years), allogeneic stem cell transplantation (8 months)	A H1N1 (27 days)	Day 0	None	H274Y (AB465342)	NP	NP	ND	ND
5	M	13	Non-Hodgkin lymphoma (34 months)	A H3N2 (32 days)	Day 4	None	None (AB462351)	None (AB462373)	None (AB462350)	0.32	0.57
4	M	19	Anaplastic large cell lymphoma (10 years), allogeneic stem cell transplantation (33 months), donor lymphocyte infusions (12 months)	B (14 days)	Day 0	None	ND	NP	. . .	ND	ND
3	M	57	Non-Hodgkin lymphoma (2 months)	A H3N2) (55 days)	Day 1	None	None (AB462363)	None (AB462362)	S31N (AB462364)	ND	ND
					Day 22	None	None (AB462366)	L111L/I (AB462365)	S31N (AB462367)	ND	ND
					Day 55	None	ND	ND	ND	ND	ND
2	M	47	Non-Hodgkin lymphoma (10 years), allogeneic stem cell transplantation (16 months), donor lymphocyte infusions (11 months)	A H3N2 (64 days)	Day 0	None	None (AB462355)	None (AB462354)	S31N (AB462356)	ND	ND
					Day 7	Oseltamivir	E119V, R292K (AB462358)	A138S (AB462357)	S31N (AB462359)	94	3.3
					Day 38	None	None (AB462360)	ND	S31N (AB462361)	ND	ND
					Day 64	None	ND	ND	ND	ND	ND
1	F	0	Severe combined immunodeficiency (3 months)	A H3N2 (275 days)	Day 0	None	None (AB462526)	135T/A, 189N/K (AB462525)	None (AB462527)	0.56	1.2
					Day 78	Oseltamivir	R292K (AB462529)	H56Y, T135A, N189K (AB462528)	A30S (AB462530)	4382	26
					Days 85 and 89[d]	Oseltamivir	E119V (AB462532) (AB462535)	T135T/A,D188D/N N189N/K (AB462531) (AB462534)	None (AB462533) (AB462536)	Range, 44–62	Range, 1.0–1.4
					Days 152, 173, and 244[d]	None	E119V (AB462538) (AB462541) (AB462543)	H56H/Y, Q57Q/R, Y94Y/H, T128T/A, T135T/A, A138A/S, D188D/N, N189N/K (AB462537) (AB462540) (AB462542)	None (AB462539) (AB462544)	Range, 46–50	Range, 0.96–1.0
					Day 260	Oseltamivir	E119V (AB462546)	Y94Y/H, T128T/A, D188D/N (AB462545)	None (AB462547)	53	1.1

NOTE. HA1, hemagglutinin 1; M2, matrix 2; NA, neuraminidase; ND, no data obtained because of primer mismatch, poor viral growth, or insufficient NA activity; NP, test not performed.

[a] Duration of continuous influenza virus excretion demonstrated by polymerase chain reaction.

[b] NA and M2 mutations associated with antiviral resistance.

[c] HA1 mutations were compared with the precursor virus.

[d] Combined sequence and antiviral resistance results for multiple viruses are depicted.

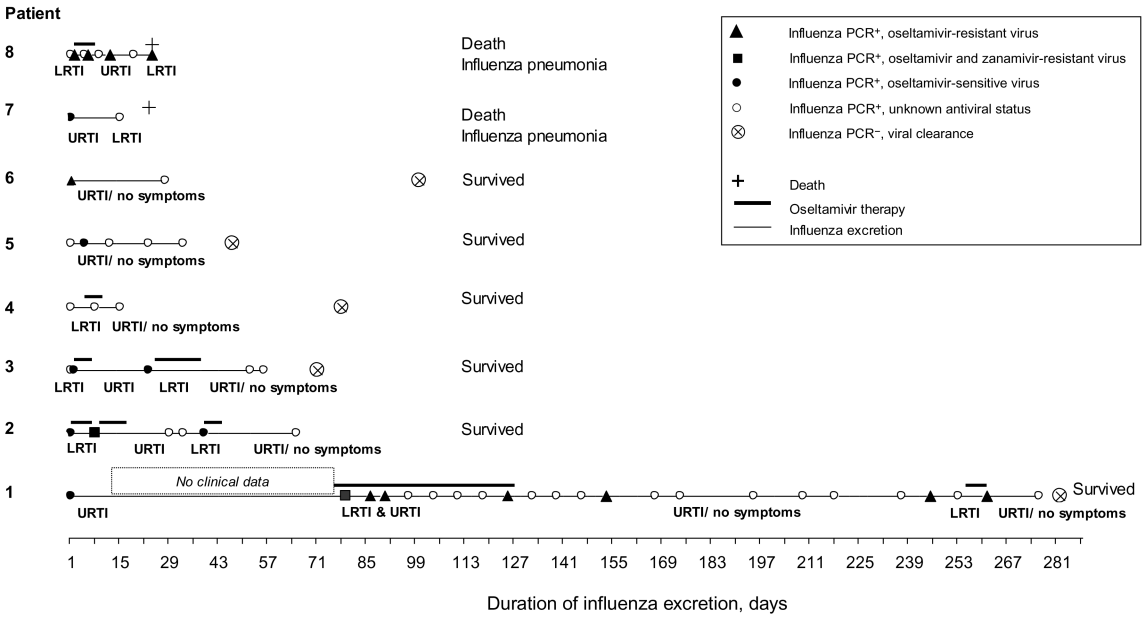

Figure 1. Time line for patients with prolonged excretion of influenza virus. Patient numbers correlate with numbers depicted in table 1. Day 0 correlates with the day influenza virus was initially detected by culture or polymerase chain reaction (PCR). Data on antiviral therapy were lacking for many time points when no viral isolate or RNA nucleotide sequence could be obtained because of low viral levels and no viral growth. LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection.

Detection of antiviral-resistant viruses. Five of 8 patients were treated with oseltamivir (figure 1). Two of the 5 (patients 4 and 8) were excluded from resistance analysis because of low viral levels and no viral growth or because of NAI-resistant precursor influenza virus. Among the 3 patients for whom resistance analysis was performed (patients 1–3), NAI-resistant viruses emerged in patients 1 and 2. In patient 1, an influenza A (H3N2) NA gene R292K escape mutant emerged during therapy, with a >7000-fold increase in resistance to oseltamivir and a >20-fold increase in resistance to zanamivir, compared with precursor virus (table 1). This virus reverted to an NA gene E119V mutant virus that had ~100-fold reduced susceptibility to oseltamivir compared with precursor virus but was sensitive to zanamivir (table 1). Continuous E119V virus excretion was confirmed for 6 weeks during oseltamivir therapy and for >4 months after cessation of antiviral therapy. Sequence analysis during follow-up revealed transient HA gene T/A135T/A, A138S, D188D/N, and N/K189N/K substitutions at or near the receptor binding site compared with precursor virus.

In patient 2, a mixed population of influenza A (H3N2) NA gene R292K (10%) and NA gene E119V (90%) escape mutants with HA gene A138S substitution at the receptor binding site emerged during therapy (table 1). Because precursor virus isolation in cell culture was unsuccessful, possibly owing to low viral load, it was not possible to generate precursor IC_{50} values. Nevertheless, HA and NA sequences derived from the precursor clinical specimen were free of known resistance markers and were very similar to those of seasonal A (H3N2) viruses. Therefore, the IC_{50} of the antiviral resistant A (H3N2) virus isolate that emerged could validly be compared with its baseline NAI susceptibility and was ~300-fold for oseltamivir and ~6-fold for zanamivir. The mixed population of NAI-resistant viruses subsequently reverted to sensitive wild-type virus 3 weeks after discontinuation of oseltamivir.

Influenza A M2 gene mutations conferring adamantane resistance were detected in samples from 3 of 6 patients (patients 1–3). The influenza A (H3N2) M2 gene S31N mutations observed in samples from patients 2 and 3 were preexistent and genetically stable, in contrast to the newly acquired A30S mutation that subsequently reverted to adamantane-sensitive virus in patient 1.

Clinical outcome and viral clearance. Six (75%) of 8 patients had 10 episodes of influenza lower respiratory tract infection (figure 1), and 4 patients required mechanical ventilation. Oseltamivir treatment was associated with clinical improvement of lower respiratory tract infection (8 of 8 episodes; patients 1–4 and patient 8), compared with untreated episodes in patients who died of influenza pneumonia (improvement in 0 of 2 episodes; patients 7 and 8) ($P = .02$, by Fisher's exact test). Influenza lower respiratory tract infection reappeared in 4 patients after antiviral therapy and clinical improvement (figure 1). Renewed clinical deterioration was likely caused by the same influenza A (H3N2) virus in each of 3 patients, because the NA gene sequences were identical (≤0.1% mutation frequency) to those of the precursor virus. NA sequences differed significantly among the 3 patients (>0.5% mutation frequency).

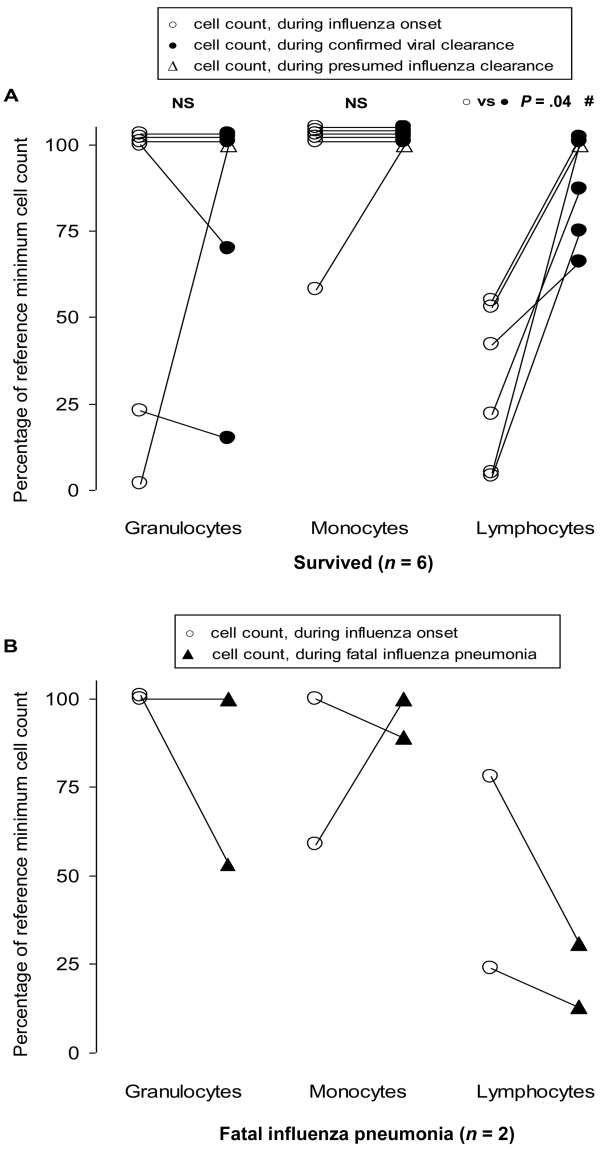

Figure 2. Cellular immune response (cell counts) during prolonged influenza excretion among survivors *(A)* and patients with fatal influenza pneumonia *(B)*. Cell count during influenza onset was obtained ⩽1 week before or after first positive polymerase chain reaction (PCR) result for influenza; cell count during confirmed viral clearance or presumed influenza was obtained ⩽2 weeks after final positive PCR result; cell count during fatal influenza pneumonia was obtained ⩽1 week before fatal influenza pneumonia. #Wilcoxon matched-pairs signed rank test revealed significant lymphocyte reconstitution during confirmed viral clearance. NS, not significant.

Confirmed viral clearance in 5 patients correlated with lymphocyte reconstitution ($P = .04$) (figure 2) and was attributed to successful HSCT (patient 1 at day 247 and patient 5 at day 22) or to spontaneous lymphocyte reconstitution (patients 3, 4, and 6). In contrast, decreasing lymphocyte counts were observed in 2 patients with fatal influenza pneumonia (figure 2). Viral clearance was never observed during the first 2 weeks after 8 courses of oseltamivir administered to 5 patients (5 regular courses of 5–6 days; 3 prolonged courses of 13–37 days) (figure 1) or after unsuccessful HSCT (day 110, patient 1). Three patients never received oseltamivir treatment (figure 1); 1 died of unrecognized fatal influenza pneumonia (patient 7), and 2 rapidly cleared the virus after lymphocyte reconstitution that followed successful HSCT (patient 5) or spontaneous lymphocyte reconstitution that followed "mild" lymphocytopenia (i.e., 55% of reference minimum cell count) at onset (patient 6).

DISCUSSION

The occurrence and clinical manifestations of prolonged influenza virus respiratory tract infection and the associated risk of developing antiviral resistance are poorly documented. This study included 8 immunocompromised patients who had symptomatic prolonged viral excretion for ⩾2 weeks, with development of influenza lower respiratory tract infection in 6 patients (10 episodes) and frequent detection of drug resistance after therapy.

The occurrence of prolonged influenza virus infection among 8 patients during a 3-year period in a hospital setting appeared to be more common than would have been expected from the few cases documented in the literature [8–12]. All 8 patients in the current study who had prolonged influenza virus infection were immunocompromised with lymphocytopenia, but a few presented with either granulocytopenia (2 of 8 patients) or monocytopenia (2 of 8 patients). This suggests that unimpaired cell-mediated immunity is important for the timely elimination of influenza viruses, a result that agrees with earlier observations [14]. In this study, complete viral clearance was associated with lymphocyte reconstitution ($P = .04$) (figure 2) and was never observed within the first 2 weeks after oseltamivir therapy (figure 1). This provides new evidence that complete influenza virus clearance depends on lymphocyte reconstitution, irrespective of receipt of oseltamivir therapy. Variable outcomes among patients who never received antiviral therapy indicate that the level and duration of lymphocytopenia could determine later outcome during prolonged influenza virus infection.

NAI-resistant influenza viruses emerged in 2 (67%) of 3 patients eligible for resistance analysis. This rate is much higher than incidences of oseltamivir resistance reported for immunocompetent adults or adolescents (0.33%–2%) and pediatric patients (4%–18%) during clinical trials [6, 7, 13]. Emergence of NAI-resistant influenza viruses was confirmed in patients 1 and 2. In patient 1, an influenza A (H3N2) virus with NA gene R292K substitution, which conferred high-level oseltamivir resistance (>7000-fold increase, compared with precursor virus) and zanamivir resistance (>20-fold increase, compared with precursor virus) (table 1), emerged from wild-type NAI-sensitive precursor virus during oseltamivir therapy during the summer months. There was no mo-

lecular variability that would suggest the presence of a mixed population. This was followed by the rapid emergence of influenza virus with NA gene E119V framework mutation, which conferred reduced oseltamivir susceptibility (~100-fold decrease), compared with precursor virus (table 1). Viral growth seemed unaffected, as observed elsewhere [24], because the E119V mutant virus was cultured from the patient's samples for 6 weeks during oseltamivir therapy and for >4 months after cessation of antiviral therapy (figure 1).

Although amino acid substitutions and subpopulations were detected in HA, NA, and M2 genes, sequence comparison of the viruses 244 and 260 days after infection with the precursor virus revealed only 1 fixed nucleotide substitution in both HA and NA genes (NA gene E119V). In addition, 260 days after the onset of infection in patient 1, the Dutch 2005–2006 influenza epidemic had started, caused by a significantly deviated influenza A (H3N2) strain. Reinfection with a new influenza A (H3N2) strain is therefore unlikely, a conclusion further supported by the fact that a single virus sequence was obtained during the summer, when there was no circulation of influenza virus in the community. The E119V mutant virus likely retained its pathogenicity during continuous influenza excretion, illustrated by renewed clinical deterioration to viral lower respiratory tract infection after a 4-month period of upper respiratory tract infection or no symptoms. Transient T/A135T/A, A138S, D188N, and N/K189N/K amino acid changes at or near the HA receptor binding site, compared with precursor virus, may have contributed to persistence of the E119V variant, partially compensating for potential reduced NA activity by a decreased HA affinity [25]. HA gene receptor binding site alterations may emerge and persist more easily among severely immunocompromised patients, with continuous high-level viral replication and decreased immunogenic selection control among viruses with aberrant HA antigens. R292K variants appear impaired, as described elsewhere [24], because this mutation did not persist in the patient and was possibly selected out by overgrowth of the E119V variant.

In patient 2, a mixed population of NA gene R292K (10%) and NA gene E119V (90%) mutants with HA gene A138S substitution at the receptor binding site and reduced NAI susceptibility emerged during oseltamivir therapy and reverted back to wild-type NAI-sensitive virus after antiviral treatment was discontinued. The emergence of R292K substitution in viruses from patients 1 and 2, which confers high-level oseltamivir resistance and reduced zanamivir susceptibility, is of concern because these mutants hamper therapy with NAIs. Apparently, with selective pressure and lack of immunological containment, NAI-resistant minor variant viruses can emerge that retain their replicative ability and evade antiviral therapy. The cessation of oseltamivir therapy allowed reversion to sensitive wild-type influenza virus A (H3N2) in patient 2, a result consistent with prior findings [8, 9] and in contrast to the result observed for patient 1, in whom the E119V mutation remained fixed after cessation of oseltamivir therapy and until viral clearance.

None of the patients received adamantane therapy; it was considered unreliable because of the rapid development of resistance [2, 3]. The M2 protein S31N substitution in viruses from patients 2 and 3 was not unexpected, because most 2006 national sentinel influenza A (H3N2) viruses also displayed this amino acid substitution (data not shown). The detected M2 protein A30S substitution in patient 1 was apparently acquired by spontaneous mutation and therefore unrelated to exposure, as has been proposed earlier for the S31N mutation [5]. A30S mutation has been observed in vivo in avian influenza virus A (H7N2) field isolates [26]. M2 protein mutations at position 30, conferring amantadine resistance are typically A30V or A30T in humans [27]; we are not aware of documented A30S mutation in human influenza viruses.

Lymphocytopenia at the time of infection has been documented as an important risk factor for developing influenza virus pneumonia [20]. In this study, the occurrence of influenza lower respiratory tract infection in 6 (75%) of 8 patients and the subsequent fatal outcome in 2 (33%) of 6 patients was similar to the rates of infection and death (80% and 33%, respectively) reported among severely immunocompromised patients elsewhere [28]. Clinical improvement of influenza lower respiratory tract infection, in which it became upper respiratory tract infection or symptoms ceased was significantly associated with oseltamivir treatment (8 of 8 episodes) compared with untreated episodes (0 of 2 episodes) ($P = .02$). During continuous influenza virus excretion, the remarkable reappearance of specific viral lower respiratory tract infection symptoms was observed in 4 patients (patients 1, 2, 3, and 8). This affirms the importance of timely recognition and treatment of influenza lower respiratory tract infection among severely immunocompromised hosts.

We conclude that prolonged influenza virus respiratory tract infection is observed among immunocompromised patients with lymphocytopenia and is associated with frequent development of influenza lower respiratory tract infection and antiviral resistance during therapy. PCR-based monitoring for this category of patients should enable the early detection of influenza viruses and could help prompt the initiation of timely therapeutic and preventive measures. Lymphocyte reconstitution is associated with viral clearance, irrespective of receipt of antiviral therapy, a result that warrants further exploration of therapeutic approaches that aim to improve specific immune recovery during prolonged influenza infection. These strategies are important for the improvement of individual outcomes and for helping to prevent the emergence and continuous excretion of drug-resistant viruses.

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