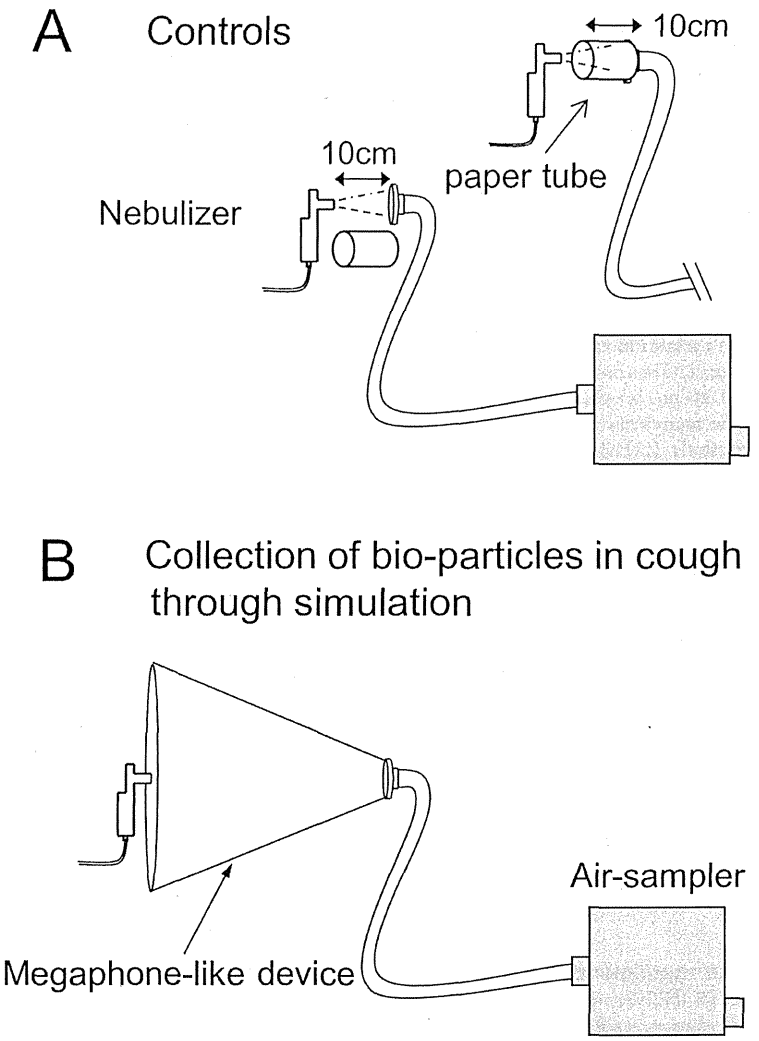

Figure 2. A schematic diagram of simulation experiments to collect biomist particles. Settings for the experiments to simulate biomist collection from coughs to estimate the viral recovery rate of this system. Control experiments with mist particles generated from a nebulizer were drawn directly into the gelatin filter membrane, with or without a hollow paper tube to guide the mist to the membrane (A), and the collection of the particles through the cone-shaped device to guide particles expelled through a cough (B).
doi:10.1371/journal.pone.0103560.g002

collection of mist by the membrane might be drastically reduced by the diffusion of the mist, and the result was compared with that of an experiment conducted without the use of a tube. Then, the cone-shaped device employed for cough collection was attached, the nebulizer was placed at the entrance of the device, and the viral mist collection experiments were repeated. (Figure 2 B). We observed that the generated mist was smoothly sucked into the cone-shaped device. After mist collection, the gelatin membrane was dissolved in MEM and the viral M1 gene copy-number was quantified using qRT-PCR. The results showed that the recovery rates of the viruses between the controls with and without the tube were almost equal and the results of the system employing the cone-shaped device were approximately 58% of the controls. We obtained similar results when less concentrated viral fluid (10^4 PFU/mL) was atomized (Table 1).

To estimate the recovery rate of the quantification process, we measured the amount of viral RNA in each step (Table 1). Briefly, 10 μL of viral fluid was poured into a gelatinous solution composed of the membrane dissolved in 10 mL of MEM. The solution was treated with collagenase to digest the gelatin to prevent regelatinization and diminish the viscosity to ultimately improve the efficiency of viral condensation in the subsequent ultracentrifugation step. The viral precipitate obtained with ultracentrifugation was subjected to RNA extraction and concentrated by freeze-drying and vacuum-centrifugation. Our results showed that the subtotal recovery rate of the quantification process was estimated at about 20%, and the total recovery rate of our system was $\geq$10%.

Table 1. Viral recovery rate of collection and quantification system.

The recovery rate in collection system using a megaphone-like device				
Setting for collection of the mist	Experiment 1 (high dose)[a]		Experiment 2 (low dose)[b]	
	Viral load[c] (copies)	Recovery rate[d] (%)	Viral load (copies)	Recovery rate (%)
Controls: close to membrane[e] with hollow paper tube	3.08×10^7 (SE 1.20×10^7)	100	NT	NT
without the tube	3.11×10^7 (SE 2.63×10^6)	100	5.9×10^3	100
With a megaphone-like device[f]	1.80×10^7 (SE 1.72×10^6)	58	3.2×10^3	54
The viral recovery rate in quantification process				
Steps in the quantification system	Experiment 3 (high dose)		Experiment 4 (low dose)	
	Viral load (copies)	Recovery rate[g] (%)	Viral load (copies)	Recovery rate (%)
Starting materials[h]	4.4×10^7	100	1.9×10^5	100
Membrane dissolution[i]	3.6×10^7	82	1.6×10^5	84
Collagenase treatment[j]	4.0×10^7	91	1.9×10^5	100
Concentration[k]	9.4×10^6	21	3.6×10^4	19

[a]The experiments were performed in triplicate for the high amount experiment controls and the number is the average and standard error (SE) of the results.
[b]The low dose experiment was performed once to confirm the reproducibility of the recovery rate, as determined by the high dose experiment.
[c]Average copy number of the influenza A M1 gene in the gelatin membrane.
[d]Percentage of viral load on the gelatin membrane to that of collection when the nebulizer was set close to the membrane.
[e]The nebulizer was set 10 cm from the gelatin membrane filter through which the viral mist was suctioned.
[f]The cone-shaped, megaphone-like device of 50 cm in length was connected with the gelatin membrane filter and the nebulizer was set at the entrance of the device.
[g]Percentage of viral load in the product of each step to those at the start of each step.
[h]Viral load in the viral fluid poured into the gelatin membrane solution.
[i]Viral load in the solution of the gelatin membrane filter dissolved in 10 mL of MEM.
[j]The gelatin solution containing the virus was digested with 10 μg/mL of collagenase.
[k]The digested fluid was ultra-centrifuged at 125,000 g for 100 min and the viral RNA was extracted from the precipitate and concentrated by freeze-drying and vacuum centrifugation.
doi:10.1371/journal.pone.0103560.t001

Quantification of viral loads in coughs of influenza patients

To apply the above system in actual clinical setting, we collected particles from coughs of 56 influenza patients, whose diagnosis was confirmed by viral isolation from throat swabs. The average patient age was 25.4 ± 10.5 years (range, 5–67 years), 88% of the samples were from 18–38-year-old patients, and the male-to-female ratio was 3:1. None of the patients had significant health problems. Forty-five (80%) cases were infected with influenza A(H1) virus, 5 (9%) with A(H3), and 6 (11%) with A(H1N1)pdm09 (Table S1).

Next, the amount of viable viral particles trapped by the gelatin membrane was quantified using a conventional plaque assay. Viable viruses were detected in 3 (5%) samples with titers of 1.5, 1.5, and 18 PFU per cough of viral subtypes A(H1), A(H3) and A(H1N1)pdm09, respectively. Due to the potential existence of non-viable virions, qRT-PCR targeting a viral gene was simultaneously performed. The detection limit for reliable quantification in our qRT-PCR system was preliminary determined as 10 gene copies per μL cDNA of the influenza virus gene. Calculations with dilution rates by the volume of the membrane solution and the times of coughs, as well as results of our actual cases, determined that the detection limit was about 10 copies per cough. Using this detection limit as a parameter, the cases were deemed as either positive or negative for the presence of influenza virus. Of the 23 (41%) cases determined as positive, and the viral load varied widely from 10 to 2240 copies/cough. Seventeen (30%) samples contained a few to several dozen copies/cough, five (9%) contained 100–200 copies/cough, and one contained an exceptionally high level, i.e., 2240 copies/cough; the remaining samples had an average and median of 63 and 44 copies/cough, respectively, with a standard deviation of 56. The highest viral content was found in a sample obtained at day 3 of illness in a patient with the influenza A(H1N1)pdm09 virus infection, which was one of three cases in this study in whom active virus was detected (the plaque count = 18/cough).

The results were analyzed from different perspectives depending on the characteristics of individual cases and initially graphed with the days of illness along the abscissa. Day 1 was defined as the onset day of any influenza symptom (e.g., high fever and coughing). We did not find any significant differences in the viral detection rates among groups of viral subtypes (Figure 3, Table S1), influenza seasons (Table S1, Figure S2), or gender (Table S1). Moreover, we did not find any specific tendency related to patient body temperature (Figure S3).

Viral loads in coughs from patients following vaccination and/or antiviral treatment

For infection control, it is important to determine the presence or absence of vaccination and antiviral treatment effects on viral load. Therefore, we analyzed the obtained data to assess the impact of patient vaccination and antiviral treatment status on the viral load in a cough. Among 56 cases, 11 (20%) received influenza vaccinations before the influenza season and 34 (61%) received antiviral treatments with the neuraminidase (NA) inhibitors oseltamivir or zanamivir at the time of cough collection.

First, we graphed the viral loads in individual cases along with the state of antiviral treatment against time from treatment initiation to cough collection (Figure 4). Here we plotted the data of days 2 and 3 together because there was no clustering of vaccination or antiviral treatment status among cases over these days.

For cases treated during a supposedly sufficient time for viral replication before cough collection (>24 h) in the host, the

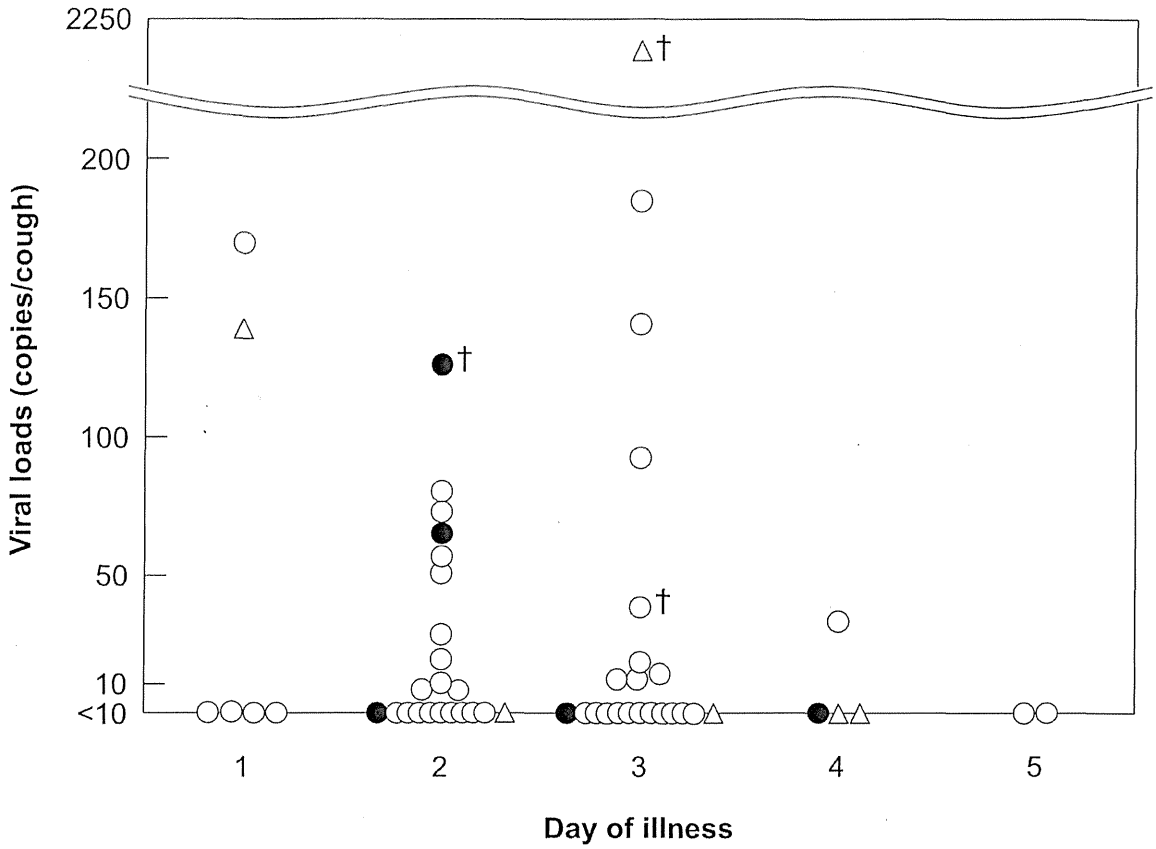

Figure 3. Viral loads in coughs of influenza patients. Cough samples were collected from individual patients and subjected to viral quantification. The viral load/cough was plotted on the ordinate along with days of illness during sample collection on the abscissa. The onset day of any influenza symptom was defined as day 1. Viral subtypes are presented using different symbols: open circle, closed circle, and open triangle represent infections with A(H1), A(H3), and A(H1N1)pdm09 virus, respectively. Daggers indicate cases in which the active virus was detected using a conventional plaque assay.
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detection rate was similar among control untreated cases (3/6, 50% vs 4/7, 57%) as was the viral load ($p = 1.00$, Fisher's exact test; $p = 0.73$, Mann–Whitney U test). We were also interested in the results of patients with a shorter duration of treatment (1.5–4.5 h) from the perspective of whether viral release from the respiratory tract by coughing could be inhibited by drug administration after the viral budding process concluded. This point of view was based on the mechanisms of the action of NA inhibitor drugs, as the timing of this action is considered important at the moment of viral release at viral budding sites to inhibit release by the inactivation of the viral NA enzyme. Thus, we wished to determine whether there was another mechanism by which the drugs enhanced rebinding of the virus to the cell surface, which had already been released from the cell surface, and thus reduce the viral load of the cough or not. We, therefore, added the results of 10 such cases to the same graph and found that the virus detection rate of these cases (5/10, 50%) was also similar to those of both untreated and sufficiently treated cases (both $p = 1.00$, Fisher's exact test), as was the viral load ($p = 0.33$ and 0.65, Mann–Whitney U test, respectively). However, when the medians were compared, these short-term treated cases showed no significant difference with the untreated cases, but the sufficiently treated cases showed apparently smaller viral loads (median = 50.6, 72.8, and 19.7 particles/cough, respectively).

On the other hand, we analyzed cases that did not receive antiviral treatment, by the status of receiving vaccination of seasonal vaccines before onset of seasonal influenza epidemic of subtype H1N1 or H3N2. Viruses were detected in one (17%) of six vaccinated cases and in four (67%) of the six control unvaccinated cases. The viral loads of vaccinated cases were lower than those of the unvaccinated cases (Figure 5 A). These results suggest that vaccination has the potential to suppress viral spread through coughing, although there was only a marginally significant difference in the viral load and virus detection rate results, possibly because of the small sample size (the median viral load = 12.2 and 72.8 for with and without vaccination; $p = 0.07$, Mann–Whitney U test). This tendency was similar even when cases that received antiviral treatment were included for analysis (the median viral load = 12.2 and 44.5, respectively; $p = 0.18$, Mann–Whitney U test) (Figure 5 B).

Comparison of viral loads in coughs from patients with and without the symptom of cough

In this study, cough samples were collected from patients by voluntary coughing. We thought that it would be important to determine whether the symptom of cough impacted that actual viral loads in a cough; therefore, we considered coughing by non-symptomatic patients, by e.g., due to allergy, physiological

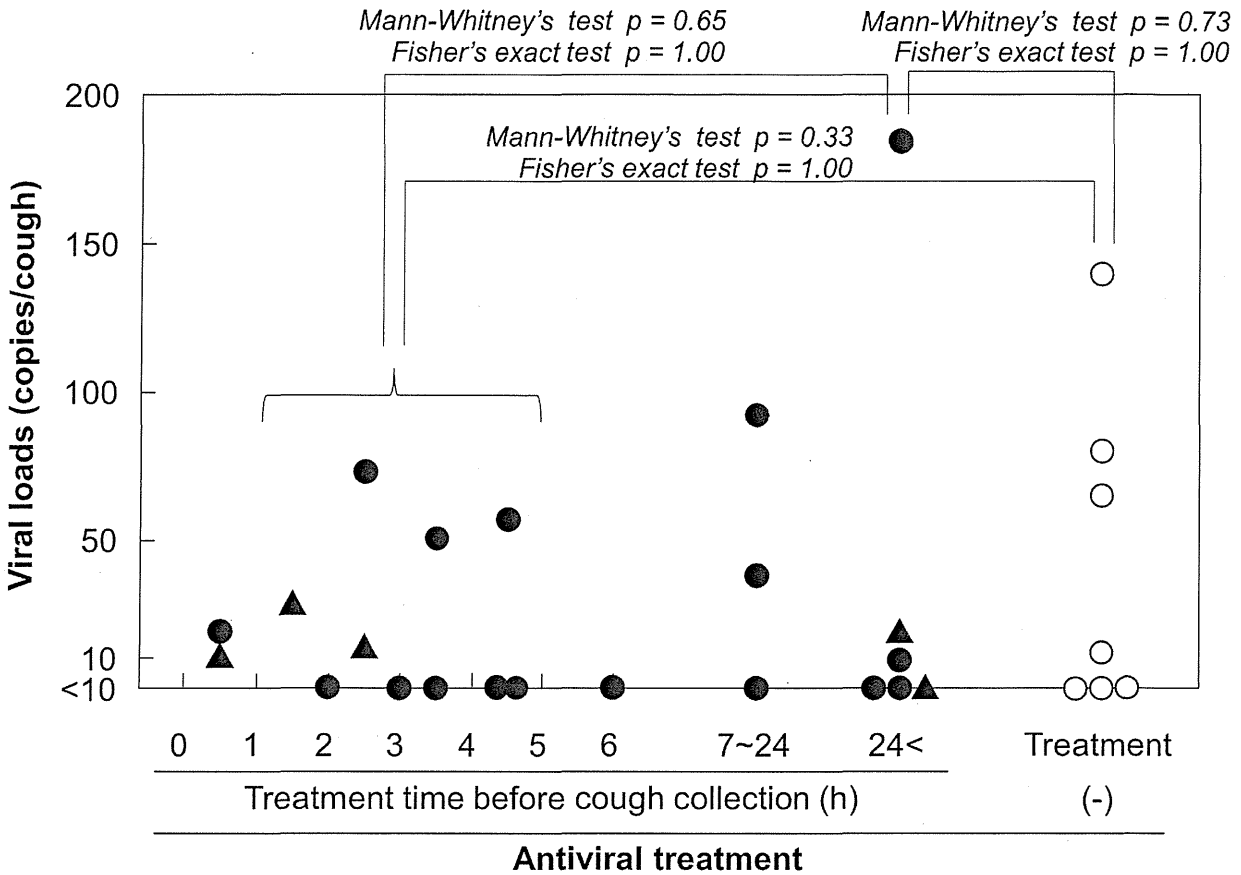

Figure 4. Viral loads in coughs of unvaccinated influenza patients. Data from days 2 and 3 of symptom onset were plotted together in respect to the status of receiving antiviral treatment, including treatment time before cough collection. The open circle, closed circle, and closed triangle represent subjects untreated, and treated with oseltamivir or zanamivir, respectively. Viruses isolated in oseltamivir-treated cases were all subtype A(H1), which is considered to be oseltamivir-resistant.
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stimulations, or causes other than influenza infection. Viral loads in the cough samples collected by voluntary coughing were compared between influenza cases with and without a symptom of coughing using throat swab samples as control (Figure 6). The viral loads in the throat swabs were calculated from the viral load of the transport medium. The approximate quantity of the collected swab fluid was estimated by the increase of the weight of the cotton swab used for sample collection, which was based on the tentative assumption that the specific gravity of the sample was 1.0 and the sample absorbed in the cotton swab was almost completely washed out into the transport medium. The viral load of the cough in patients with the symptom of cough was higher than for those without it (median = 53.7 and 38.3, respectively). However, the difference was not statistically significant ($p = 0.33$, Mann–Whitney U test), as was seen with throat swab (median = 5644 and 6654, respectively; $p = 0.79$, Mann–Whitney U test). However, interestingly, several patients in the symptomatic group had higher viral loads than in the non-symptomatic group. In addition, we also found that the cases with the highest viral loads in the throat swab (plot numbers 1 and 2) had undetectable levels of viruses in the cough samples; thus, the highest viral load in a cough sample (plot numbers 4–6) did not necessarily correspond with the highest viral load in a throat swab (Figure 6). Hence, our results suggest that the viral load in the cough samples, from at least those cases, might have been affected by the status of the symptom of cough, and do not necessarily directly reflect the status of viral load on the pharyngeal wall.

Relationships of viral loads in coughs, throat swabs, and oral secretions

In light of these interesting results, we further investigated whether the viral load of cough sample, as collected with our system, reflects those of the pharyngeal walls, oral secretions, or other sources, in relation to from where the virus mainly originated. We plotted combinations of viral loads obtained from cough samples and throat swabs or oral secretions of individual patients in two-dimensional graphs to examine possible correlations (Figure 7 A, B). The Spearman's rank correlation coefficient was also calculated for both combinations. The results showed only weak correlations between both cough samples/throat swabs ($r_s = 0.355$, $p = 0.007$) and cough samples/oral secretions ($r_s = 0.528$, $p = 0.00003$). Notably, several cases showed almost no correlations: a high viral load in the throat swab or oral secretion while very low or undetectable in the cough sample, and vice versa (Figure 7 A, B). These results suggest that in some cases from whom the virus was collected from a cough, at least in cases with the highest viral load with very low viral loads in the throat

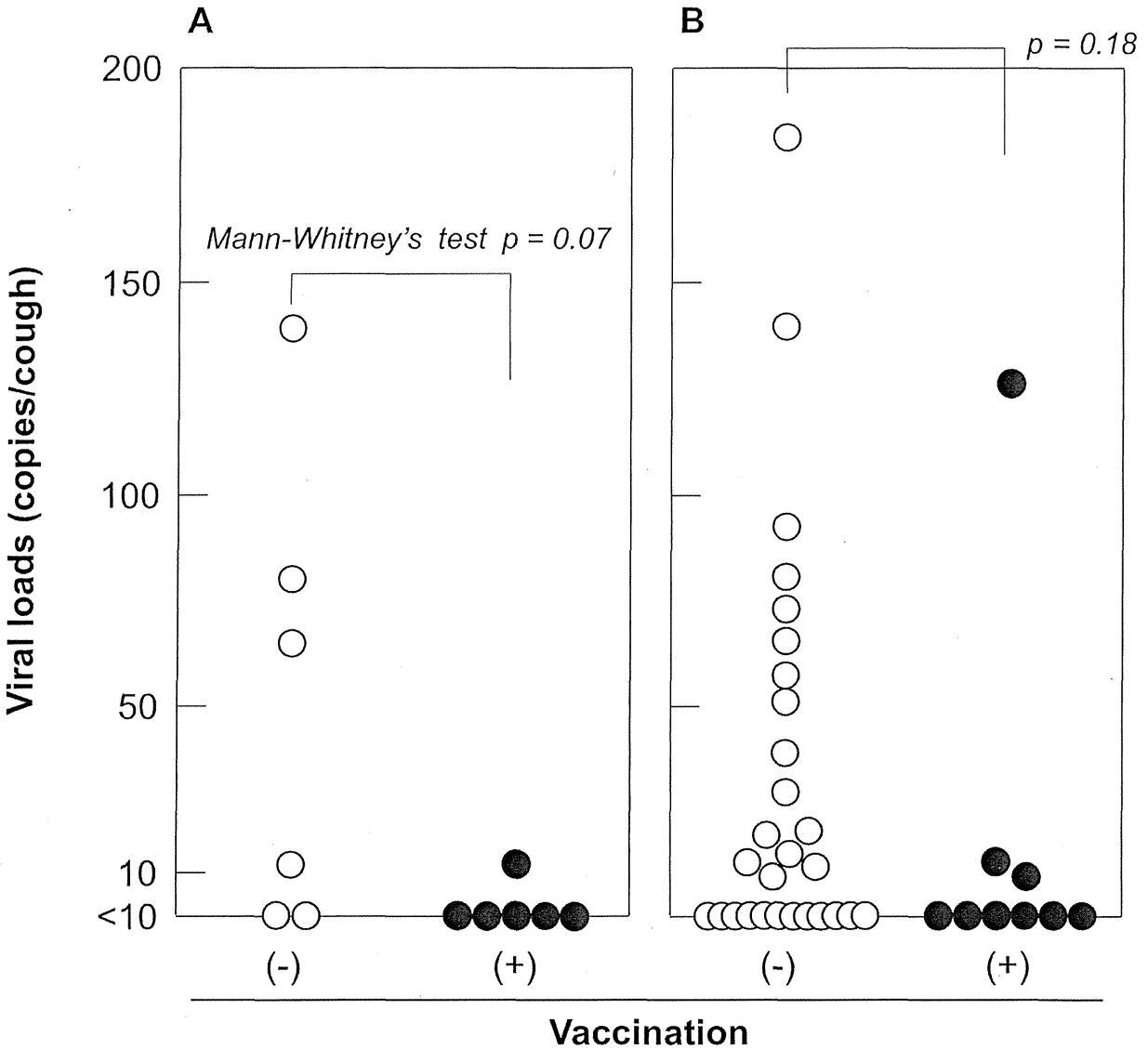

Figure 5. Viral loads in coughs of vaccinated and unvaccinated influenza patients. Viral loads were plotted against vaccination status. Data from days 2 and 3 of cases infected with A(H1) or A(H3) virus are shown together. Viral loads in coughs of antiviral-untreated influenza patients (A) and those of all cases including antiviral-treated cases (B). Open and closed circles represent unvaccinated and vaccinated cases, respectively.
doi:10.1371/journal.pone.0103560.g005

swab or oral samples, the virus in the cough might not have originated from either the throat wall or oral cavity.

Discussion

The main objective of this study was to develop a simple and portable system to collect and quantify the viral load in a cough with aim to better understand airborne infections. In this regard, we concluded that our system performed well and can be successfully applied to actual influenza patients.

In this system, we employed a gelatin membrane filter to trap viral particles. A review article of aerosol sampling reported that the efficiency of the physical collection of gelatin filters for airborne particles exceeded 96% [17]. Fabian et al. [18] reported that a recovery rate of atomized viral genes using a gelatin filter was 63% of an optimized collection system using the SKC biosampler, but the rate decreased to only 10% for infectious viruses. In the present study, viable viruses were detected in only three samples using a plaque assay. It is possible that the most of trapped viruses became inactivated while trapped in the gelatin filter until the assay was performed.

The overall viral gene recovery rate of our system was estimated at approximately $\geq$10%, suggesting that the actual viral load in a cough may be 10-fold greater than our actual measured amount, or even greater if we took into account the possible undetected losses during RNA extraction and cDNA synthesis.

In designing the cone shaped, megaphone-like device, we adopted a length of 50 cm to make the airflow velocity between the distal end of the cough and the suction speed of the sampler to ensure optimal air-sampling efficiency. However, we noticed a

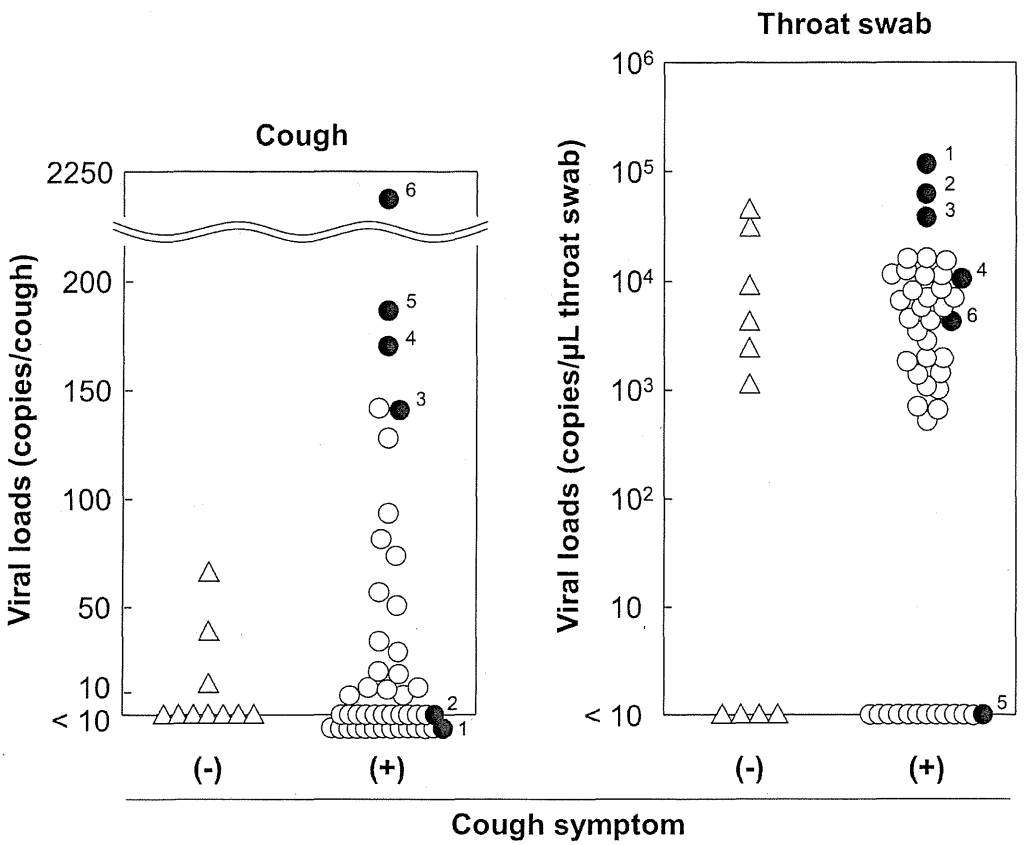

Figure 6. Viral loads of samples from patients with or without cough symptom. Viral loads in cough and throat swab samples from cases with and without a symptom of coughing were compared. The viral load in the throat swab was calculated from the viral load of the transport medium in which the swab fluid was eluted and the volume of the eluted swab fluid, which was tentatively estimated by the increase of the weight of the cotton swab used for sample collection. Circle and triangle represent cases with and without symptoms of coughing. Closed circles indicate cases with higher viral loads among cough or throat swab samples, and identical numbers associated with the closed circles indicate that the data were from the same patient.
doi:10.1371/journal.pone.0103560.g006

reflection flow in some actual cases. A stronger cough flow might collide against the inside wall, filter membrane, and/or internal air mass of the device and rebound, or flushed out the internal air mass. However, the result of our preliminary experiments showed that the spill over caused by the outward flow might not affect the result. When a volunteer smoker coughed into the device once in a chamber after one breath of smoking, the smoke particle count adjacent to the outer surface of the device was about 20-fold lower with pumping than without (data not shown). The levels of viral genes measured from coughs in many patients by our system were similar to those measured using the methods of Lindsley et al., who perfectly collected cough samples by asking the patients to blow into a spirometer [8], which may offer supporting evidence to validate the collection rate of our system.

Furthermore, we used universal primers for cDNA synthesis from the viral RNA aimed to quantify the RNA, because we were also preparing to assess influenza B cases during this study period and, therefore, could not use a carrier virus in the ultracentrifuge process. The combined use of specific primers for each viral target and some carrier viruses may improve viral recovery; however, this possibility should be examined in a future study.

Our particle collection method may not sufficiently distinguish large droplets that directly hit the membrane from the small particle droplet nuclei floating inside of the cone, probably because the 50-cm distance from the face to the filter may not long enough to exclude collection of large droplets as well. This possibility was already taken into consideration. Our method was aimed to obtain information regarding the approximate total amount of virus released by coughing.

Apart from the issue of absolute recovery rate, relative comparisons of viral loads among patients may be possible. The highest viral load was 2240 copies/cough among the 56 samples, which was more than 10-fold greater than the average of the virus-positive samples. A possible factor that may play a role in spread of infection is the existence of the so-called "super spreader," which has been proposed in epidemics of severe acute respiratory syndrome [19] and influenza [20,21]. Moreover, Lindsley et al. [8] reported a case among a total of 58 suspected influenza cases with a discharge of 355 copies/cough, and Bischoff et al. [9] also reported that 19% of emitters acted as "super emitters," which was in accordance with the ratio proposed by Lloyd-Smith et al. [20].

Here, we measured the viral loads in cough samples collected from influenza patients. However, there were some limitations to our study regarding the study subjects: most were generally healthy young adults or adolescents, and data from young

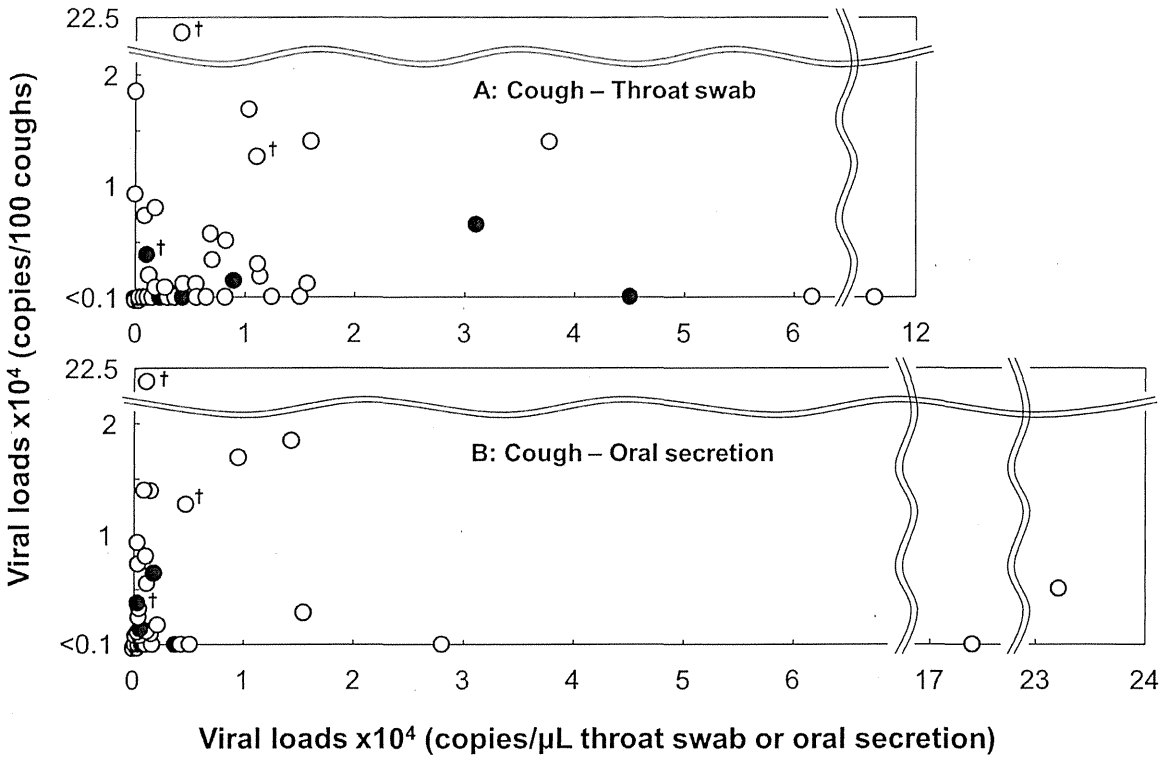

Figure 7. Viral loads of coughs compared to those in throat or oral samples in individual patients. The combined data of viral loads of the cough and throat swab, or cough and oral secretion samples of individual patients were plotted on two-dimensional graphs. The open and closed circles represent cases with and without symptom of coughing, respectively, and daggers indicate cases in which active virus was detected in cough samples using the plaque assay.
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children, elderly persons, or persons with chronic medical conditions were limited. Second, we could not follow the time course of the viral shedding in the cough among individuals.

We described applications of our methodology to study the effects of vaccination and antiviral treatment, and found that the former may suppress viral release during coughing, but the latter may not, although the former was not yet supported statistically. Regarding antiviral treatment, care should be taken when interpreting the results of this study because all cases treated with oseltamivir were infected with influenza A(H1) virus during the 2008–2009 influenza season, which was considered as resistant to this drug, reflecting the circulating virus at the time of the study [22]. However, the number of subjects included in these analyses was small and may have been insufficient to draw conclusions.

Correlation analyses on the viral loads between the cough and throat swab or oral secretion samples showed that the viral load in a cough may not necessarily reflect those of a throat swab or oral secretion and vice versa, suggesting that the virus in the collected coughs, at least for cases with significant disparities in the viral load level between the two kinds of samples, neither originated from the pharyngeal wall nor the oral cavity, and thus might be mixed with cough air flow at anatomically deeper sites in the respiratory tract than those sites. Regarding the weak correlations between samples, we cannot deny the possibility that in a certain proportion of cases, the virus might have originated from the throat wall or oral cavity as well, or that the virus in the cough may have been trapped in the throat wall and/or oral cavity and we only detected these.

In the present study, we measured the viral load mainly by genetic quantification. However, for optimal infection control, information regarding the activity of virus is more important. We detected active viruses in only 5% of cough samples with moderately high viral titers. This is probably because of the inactivation of the viruses while trapped in the gelatin membrane until plaque titration. We observed that the viruses did not become inactivated if the plaque titration was conducted within a short time after being trapped in the membrane, but they may become inactivated after an extended period with varying degrees of inactivation speed depending on the environmental temperature and humidity (data not shown). This result indicates that our methodology might be insufficient for quantification of viable viruses.

Altogether, our method can be effectively applied for point-of-care detection of influenza viruses from the cough and their quantification. Nonetheless, further large-scale studies are needed to validate our results, which involve several important epidemiological, clinical, and pathophysiological issues.

Supporting Information

Figure S1 Size distributions of bio-particles in a human cough and mist generated by a nebulizer. Comparison of the size distribution of bio-particles from human coughs and mist generated by a nebulizer. The air inside a closed 2 m^3 chamber was set at about 20% RH and cleaned-up using an air fan unit

with a high-efficiency particulate air filter unit. Immediately after healthy adult volunteers coughed voluntarily into the chamber via small window with a flap to seal it, or 2 min after the allantoic fluid of the embryonated chicken egg was atomized in the chamber for 3 s, the particle concentrations were measured using a laser particle-counter (KR-12A; Rion Co., Ltd., Tokyo, Japan) for each particle size range. The data of human cough are presented as absolute particle counts and standard error rang per single cough of the average of five subjects, and the data of the nebulizer are presented as the concentration inside the chamber.
(TIF)

Figure S2 Viral loads in coughs of influenza patients in different influenza seasons. The viral load/cough is plotted on the ordinate and the days of illness during sample collection on the abscissa. Epidemic seasons are presented using different symbols: closed circles, open circles, and open triangles represent seasons 2007–2008, 2008–2009, and 2010–2011, respectively.
(TIF)

Figure S3 Viral loads of coughs of influenza patients by body temperature. Data of individual cases are plotted on the graph with the viral load in a cough on the ordinate and body temperature at the time of cough sampling on the abscissa. Data of days 2 and 3 were plotted together in the graph. Viral subtypes are presented using different symbols: open circles, closed circles, and open triangles represent infections with A(H1), A(H3), and A(H1N1)pdm09 virus, respectively.
(TIF)

Table S1 Demographics of virus-detected and undetected cases.
(TIF)

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Author Contributions

Conceived and designed the experiments: HN SS MO. Performed the experiments: HN SS EH SO MO HY TH YS RS. Analyzed the data: HN EH TY. Contributed reagents/materials/analysis tools: SS. Wrote the paper: HN EH. Collected the clinical specimens: NH SO HY TH WS HM MS YM EH. Statistical analysis: HN EH TY.

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The Effects of Neuraminidase Inhibitors on the Release of Oseltamivir-Sensitive and Oseltamivir-Resistant Influenza Viruses from Primary Cultures of Human Tracheal Epithelium

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Defining the effects of neuraminidase inhibitors on influenza virus infection may provide important information for the treatment of patients. The effects of neuraminidase inhibitors have been examined using various methods, including viral release from kidney cells. However, the effects of neuraminidase inhibitors on viral release from primary cultures of human tracheal epithelial cells, which retain functions of the original tissues, have not been studied. The effects of neuraminidase inhibitors on the replication of the pandemic influenza virus [A/Sendai-H/N0633/2009 (H1N1) pdm09] and the seasonal influenza virus [A/Sendai-H/216/2009 (H1N1)] that was isolated during the 2008–2009 season were examined. The virus stocks were generated by infecting tracheal cells with the pandemic or seasonal influenza virus. Four types of inhibitors (oseltamivir, zanamivir, laninamivir, and peramivir) reduced pandemic viral titers and concentrations of the cytokines interleukin-6 and tumor necrosis factor-α in supernatants and viral RNA in cells. However, oseltamivir did not reduce seasonal viral titers, cytokine concentrations and viral RNA, and the 50% inhibitory concentration (IC_{50}) of oseltamivir for neuraminidase activity in the seasonal virus was 300-fold higher than that observed for the pandemic influenza virus. The seasonal influenza virus had an oseltamivir-resistant genotype. The magnitude of the IC_{50} values of the neuraminidase inhibitors for the seasonal influenza virus was inversely related to the magnitude of the inhibitory effects on viral release. These methods for measuring the release of virus and inflammatory cytokines from primary cultures of human tracheal epithelium may provide useful information regarding the effects of neuraminidase inhibitors on influenza viruses. ***J. Med. Virol. 87:25–34, 2015.***

KEY WORDS: cell culture; human airway; influenza virus; neuraminidase inhibitor

INTRODUCTION

Anti-influenza drugs include neuraminidase inhibitors, such as oseltamivir and zanamivir, which are beneficial for the treatment of patients infected with

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influenza virus [Beigel and Bray, 2008]. However, oseltamivir-resistant seasonal influenza A/H1N1 virus infection has been reported, and this type of seasonal influenza virus infection has caused severe disease in immunocompromised patients [Gooskens et al., 2009]. Increased viral replication and subsequent hypercytokinemia are associated with high pathogenicity in patients infected with influenza virus [de Jong et al., 2006]. Therefore, information is required on the effectiveness of anti-influenza drugs before treating a patient with influenza virus infection.

The effects of neuraminidase inhibitors have been examined using various methods, including evaluating influenza neuraminidase activity [Bantia et al., 1998], survival rates of infected mice, and viral titers in the lungs of infected mice and in supernatants of cultures of Madin Darby Canine Kidney (MDCK) epithelial cells [Smee et al., 2001; Itoh et al., 2009; Yamashita et al., 2009; Kubo et al., 2010]. A relationship between neuraminidase gene mutations and neuraminidase inhibitor resistance has also been reported [Kiso et al., 2004; Sheu et al., 2008; Saito et al., 2010].

Furthermore, Boivin et al. [2000] and Sugaya and Ohashi [2010] assessed influenza viral titers in pharyngeal throat swabs or secretions from patients to detect the effectiveness of oseltamivir and zanamivir, respectively. Viral RNA in nasopharyngeal swabs was also measured to study the virulence of influenza A (H5N1) [de Jong et al., 2006]. These reports suggest that data related to viral release from human airway epithelial cells, which represent the first target of influenza viral infection, may reveal more precise information about the clinical effectiveness of anti-influenza drugs and disease severity than data measured using traditional methods. Itoh et al. [2009] reported that influenza virus was released by human bronchial epithelial cells, but methods for detecting the effectiveness of anti-influenza drugs for reducing viral release from cultured human airway epithelial cells have not been established.

Primary cultures of human tracheal epithelial cells retain the functions of the original tissue [Yamaya et al., 1992], and cells cultured using this method may provide accurate information related to the effectiveness of anti-influenza drugs for the reduction of viral replication. In this study, primary cultures of human tracheal epithelial cells were infected with oseltamivir-sensitive and oseltamivir-resistant influenza viruses that were isolated from patients, and the effects of four types of neuraminidase inhibitors on viral release from the cells were examined. This study aimed to establish a new strategy for evaluating the effect of neuraminidase inhibitors on influenza virus replication in human airway epithelial cells.

To study the inhibitory effects of neuraminidase inhibitors on infection by influenza viruses that are released from human airways, stocks of influenza viruses were generated by infecting human tracheal epithelial cells with viruses.

MATERIALS AND METHODS

Human Tracheal Epithelial Cell Culture

The isolation and culture of human tracheal surface epithelial cells was performed as described previously [Yamaya et al., 2010] in a mixture of Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (DF-12) medium (Life Technologies, Carlsbad, CA) containing 2% Ultroser G (USG) serum substitute. Trachea samples for cell culture were obtained from 23 patients after death (age, 70 ± 11 yr; 11 females and 12 males). This study was approved by the Tohoku University Ethics Committee.

Culture of Madin Darby Canine Kidney Cells

MDCK cells were cultured in T_{25} flasks in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum and antibiotics [Numazaki et al., 1987; Yamaya et al., 2010]. The cells were then plated in 96-well plates and cultured.

Viral Stocks

To prepare the pandemic A/H1 2009 influenza virus [influenza A/H1 pdm 2009, A/Sendai-H/N0633/2009 (H1N1) pdm09] and the type A seasonal human influenza virus [seasonal influenza A/H1N1, A/Sendai-H/216/2009 (H1N1)], nasal swabs were collected from patients and suspended in MEM medium [Numazaki et al., 1987]. To study the inhibitory effects of neuraminidase inhibitors on the infection of influenza viruses that are released from human airways, stocks of influenza viruses were generated by infecting human tracheal epithelial cells with viruses. Cells were cultured in 24-well plates in 0.9 ml of DF-12 medium and 100 μl of MEM containing virus for 1 hr. The culture supernatants containing virus were then removed, and the cells were cultured in DF-12 medium containing 2% USG at 37°C in 5% CO_2–95% air. To obtain the influenza virus solution, the supernatants were collected and fast frozen in ethanol at −80°C.

Detection and Titration of Viruses

The detection and titration of influenza viruses in the culture supernatants were performed using the endpoint method [Condit, 2006] by infecting replicate confluent MDCK cells in plastic 96-well plates with 10-fold dilutions of virus-containing supernatants, as described previously [Yamaya et al., 2010]. After exposing the cells to the virus-containing supernatants, the supernatants were aspirated and the cells were rinsed with PBS; fresh MEM containing trypsin was then added. The typical cytopathic effects of influenza virus were then examined. The $TCID_{50}$ (TCID, tissue culture infective dose) was calculated using methods that were described previously

[Condit, 2006], and the viral titers in the supernatants were expressed as $TCID_{50}$ units/ml [Yamaya et al., 2010].

Viral Infection of Cells

Infection of human tracheal epithelial cells with influenza virus was performed using methods that were described previously [Yamaya et al., 2010]. A stock solution of influenza virus was added to the cells in 24-well plates (400 μl in each well, 1.0×10^3 $TCID_{50}$ units/ml, 0.8×10^{-3} $TCID_{50}$ units/cell of the multiplicity of infection). After a 1-hr incubation, the viral solution was removed and the cells were cultured in 1 ml of fresh medium at 37°C in 5% CO_2–95% air.

Treatment of Cells With Neuraminidase Inhibitors

The cells were treated with oseltamivir carboxylate (the active form of oseltamivir), zanamivir, laninamivir (the active form of laninamivir octanoate), or peramivir. The treatments were started 30 min prior to infection, immediately after infection or 3 days after infection and continued until the end of the experimental period after infection. To examine the concentration-dependent effects of neuraminidase inhibitors, the cells were treated with one of the four neuraminidase inhibitors at concentrations ranging from 1 nM to 1 μM.

Collection of Supernatants

When the cells were treated with neuraminidase inhibitors on day 3 after infection, the cells were infected with virus, the supernatant (300 μl) was collected 1 day (24 hr) after infection, and the same volume (300 μl) of fresh medium without neuraminidase inhibitors was added. Then, the entire supernatant volume (1 ml) was collected 3 days (72 hr) after infection; 1 ml of fresh medium containing neuraminidase inhibitors was then added, and the cells were cultured. A total of 300 μl of supernatant was collected, and the same volume (300 μl) of fresh medium containing neuraminidase inhibitors was added 2 days (48 hr) and 4 days (96 hr) after the start of treatment with neuraminidase inhibitors. The entire supernatant volume (1 ml) was collected 6 days (144 hr) after the start of treatment.

When the cells were treated with neuraminidase inhibitors immediately after infection, 1 ml of fresh medium supplemented with neuraminidase inhibitors was added after infection. The cells were then cultured, 300 μl of supernatant was collected, and 300 μl of fresh medium with neuraminidase inhibitors was added on days 1 and 3. The entire supernatant volume (1 ml) was collected 5 days (120 hr) after infection.

For treatment with neuraminidase inhibitors prior to infection, the cells were pretreated with neuraminidase inhibitors for 30 min prior to infection. The cells were also cultured in DF-12 medium supplemented with neuraminidase inhibitors during and after infection.

Quantification of Influenza Virus RNA

Viral RNA in the cells was measured to confirm the effects of the neuraminidase inhibitors on viral replication. Two-step real-time quantitative reverse transcription (RT)-PCR was performed using the TaqMan technique (Roche Molecular Diagnostic Systems, Pleasanton, CA) as described previously [Yamaya et al., 2010]. Primers and TaqMan probes for the viruses were designed as reported previously [Lorusso et al., 2010; Yamaya et al., 2010]. The expression of viral RNA was normalized to the constitutive expression of β-actin mRNA [Suzuki et al., 2002].

Assessment of Drug Susceptibility

To assess influenza virus drug susceptibility, the 50% inhibitory concentration (IC_{50}) of neuraminidase inhibitors for the viruses was determined using a fluorescence method (neuraminidase inhibition assay) [Suzuki et al., 2011].

DNA Sequencing

The sequences of the influenza viruses were determined as described previously [Suzuki et al., 2011].

Measurement of Cytokine Production

Interleukin (IL)-6 and tumor necrosis factor (TNF)-α levels in the supernatants were measured using specific enzyme-linked immunosorbent assays (ELISAs) in the measurement of IL-6 and a chemiluminescent enzyme immunoassay (CLEIA) in the measurement of TNF-α, respectively.

Statistical Analysis

The results are expressed as the mean ± SD. Statistical analysis was performed using a two-way repeated measures analysis of variance (ANOVA). Subsequent posthoc analyses were performed using Bonferroni's method. For all analyses, values of $P < 0.05$ were assumed to be significant. In the experiments that used cultures of human tracheal epithelial cells, n refers to the number of donors (tracheae) from whom the cultured epithelial cells were obtained.

RESULTS

Release of the Influenza A/H1 pdm 2009 Virus and Effects of Oseltamivir

To examine the effects of neuraminidase inhibitors on the influenza A/H1 pdm 2009 virus, human tracheal epithelial cells were treated with inhibitors 3 days after infection with the influenza virus

because treatment typically begins when patients complain of fever and visit a clinic or hospital. No virus was detected 1 hr after infection, but the virus was detected in supernatants after 24 hr and the viral titer progressively increased between 24 hr and 3 days (72 hr) after infection (Fig. 1A). The viral titer in the supernatants increased with time for the 3 days of observation ($P < 0.05$ in each case, as indicated by ANOVA), and consistent viral titers were observed for an additional 6 days (144 hr) of incubation (Fig. 1A).

When the cells were treated with oseltamivir (1 μM) 3 days after infection, the viral titers in supernatants collected 2 days (48 hr) after treatment were reduced compared to those of cells treated with vehicle alone (0.01% water), and the viral content progressively decreased 4 and 6 days after the start of treatment (Fig. 1A).

The time course of viral release from the cells treated with vehicle (0.01% water) indicated that the viral titer levels in the supernatants increased with time for the 3 days of observation, and consistent titers of the influenza A/H1 pdm 2009 virus were observed for 5 days after infection (Fig. 1B and C).

When the cells were treated with oseltamivir (1 μM) immediately after infection, significant reductions of the supernatant titers of the virus were observed on days 1, 3, and 5 after infection (Fig. 1B). Similarly, when the cells were pretreated with oseltamivir (1 μM) 30 min before, during, and after

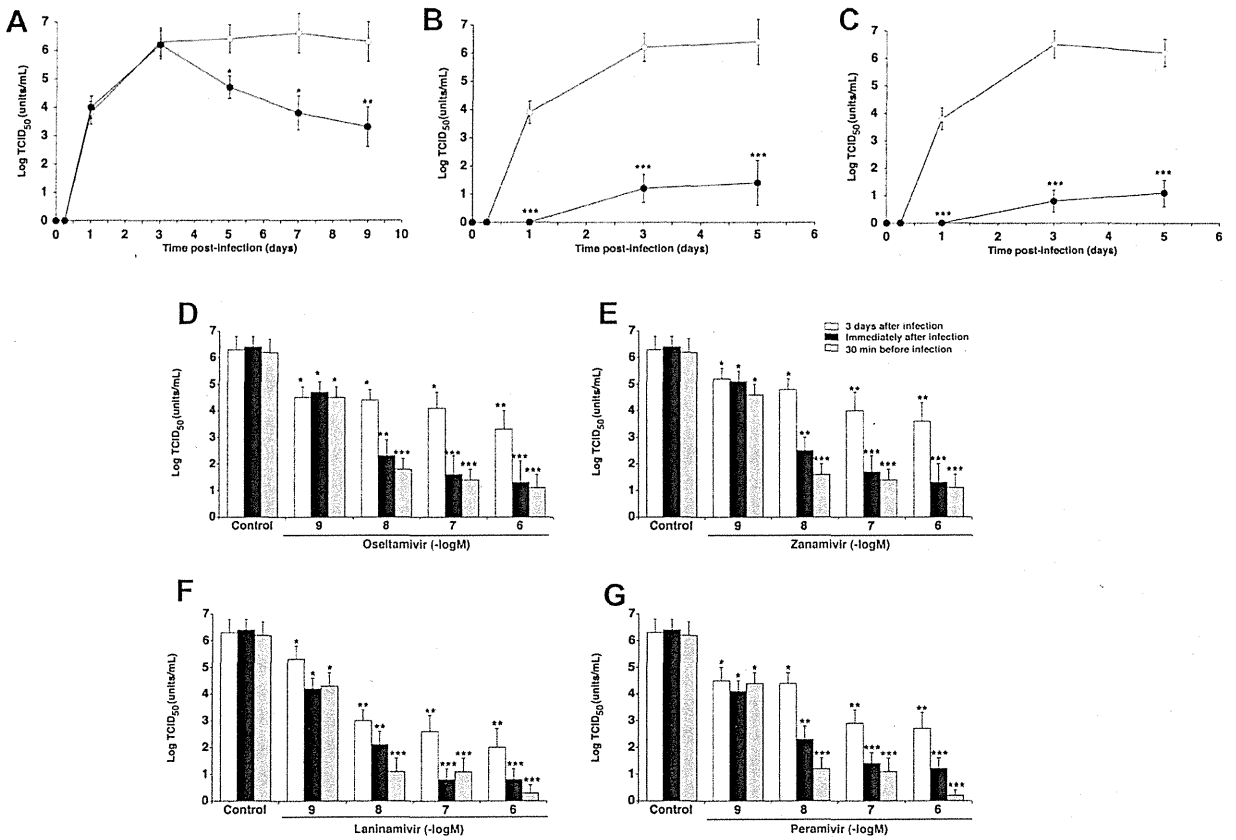

Fig. 1. **A–C**: The time course of viral release into the supernatants of primary cultures of human tracheal epithelial cells that were obtained at different times after exposure to the influenza A/H1 pdm 2009 virus in the presence of oseltamivir (1 μM; closed circles) or vehicle (0.01% water; control, open circles). Treatment with oseltamivir was initiated 3 days after infection (A), immediately after infection (B), or 30 min before infection (C) and continued until the end of the experiments. Changes in virus concentrations in the supernatants are expressed as $TCID_{50}$ units/ml. The results are expressed as the mean ± SD from five different tracheae. Significant differences compared to viral infection alone are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. **D–G**: Concentration-dependent effects of the neuraminidase inhibitors oseltamivir (D), zanamivir (E), laninamivir (F), and peramivir (G) on viral release into supernatants collected 9 days (in the culture condition of "3 days after infection") or 5 days (in the culture condition of "Immediately after infection" and "30 min before infection") after infection. Treatment with one of the neuraminidase inhibitors or vehicle (control, 0.01% water) was initiated 3 days after infection (in the culture condition of "3 days after infection"), immediately after infection (in the culture condition of "immediately after infection") or 30 min before infection (in the culture condition of "30 min before infection") and continued until the end of the experiments. Changes in the virus concentration in the supernatants are expressed as $TCID_{50}$ units/ml. The results are expressed as the mean ± SD from five different tracheae. Significant differences compared to vehicle alone (control) are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

infection, significant reductions in the supernatant titers of influenza A/H1 pdm 2009 virus were observed on days 1, 3, and 5 after infection (Fig. 1C).

Concentration-Dependent Effects of Neuraminidase Inhibitors on the Release of the Influenza A/H1 pdm 2009 Virus

When the cells were treated with oseltamivir 3 days after infection, oseltamivir decreased the viral titers in supernatants in a concentration-dependent manner; a significant reduction was observed even at 1 nM (Fig. 1D). Similarly, zanamivir, laninamivir, and peramivir reduced influenza A/H1 pdm 2009 viral titers in the supernatants in a concentration-dependent manner and significantly reduced viral titers even at 1 nM (Fig. 1E–G).

When the cells were treated with one of the neuraminidase inhibitors immediately after infection, oseltamivir, zanamivir, laninamivir, and peramivir reduced the viral titers in the supernatants in a concentration-dependent manner and a significant reduction was observed even at 1 nM (Fig. 1D–G).

Similarly, when the cells were pretreated with one of the neuraminidase inhibitors 30 min before, during, and after infection, oseltamivir, zanamivir, laninamivir, and peramivir reduced the viral titers in supernatants in a concentration-dependent manner and a significant reduction was observed even at 1 nM (Fig. 1D–G).

Effects of Neuraminidase Inhibitors on the RNA Replication of the Influenza A/H1 pdm 2009 Virus

Influenza A/H1 pdm 2009 viral RNA increased with time, and maximum viral RNA expression was observed in the cells 5 days (120 hr) after infection (data not shown). When the cells were pretreated with one of four types of inhibitors (oseltamivir, zanamivir, laninamivir, or peramivir at a concentration of 1 μM) 30 min before, during, and after infection, the inhibitors reduced viral RNA replication in the cells (Table I).

Effects of Neuraminidase Inhibitors on the Release of the Seasonal Influenza A/H1N1 Virus

The seasonal influenza A/H1N1 virus was detected in supernatants on day 1 (2.8 ± 0.4 $TCID_{50}$ units/ml, n = 3), and the viral content progressively increased between days 1 and 3 after infection (6.4 ± 0.8 $TCID_{50}$ units/ml, $P < 0.05$, n = 3). Furthermore, consistent viral titers were observed for an additional 6 days (through 9 days after infection) (data not shown). Oseltamivir did not reduce the viral titers when the cells were treated with oseltamivir (1 μM) from 3 days after infection, immediately after infection or from 30 min before infection (Fig. 2).

Zanamivir and laninamivir reduced seasonal influenza A/H1N1 viral titers at concentrations of 10 and 1 nM and at higher concentrations, and peramivir reduced viral titers at 100 nM when the cells were treated with the drugs from 30 min before infection (Fig. 3).

Effects of Neuraminidase Inhibitors on the RNA Replication of the Seasonal Influenza A/H1N1 Virus

Seasonal influenza A/H1N1 viral RNA increased with time, and maximum viral RNA was observed in the cells 5 days after infection (data not shown). Treatment of the cells with oseltamivir (1 μM) from 30 min before, during, and after infection did not reduce viral RNA in the cells (Table I). Zanamivir and laninamivir (1 μM) reduced viral RNA replication in the cells, but peramivir (1 μM) did not reduce viral RNA in the cells (Table I).

Susceptibility to Neuraminidase Inhibitors

The average IC_{50} values of oseltamivir, as indicated by neuraminidase inhibition assays, were less than 10 nM, and those of zanamivir, laninamivir, and peramivir were less than 1 nM for the influenza A/H1 pdm 2009 virus (Table II). In contrast, the IC_{50} values of oseltamivir and peramivir for the seasonal influenza A/H1N1 virus were higher than those for the influenza A/H1 pdm 2009 virus (Table II). The IC_{50} values of oseltamivir for the seasonal influenza A/H1N1 virus were more than 300-fold higher than those observed for the influenza A/H1 pdm 2009 virus.

DNA Sequencing

The seasonal influenza A/H1N1 virus had a histidine-to-tyrosine amino acid substitution at position 275 (H275Y mutation) of the neuraminidase gene, but the influenza A/H1 pdm 2009 virus did not have this mutation (Table II).

TABLE I. RNA Replication of Influenza Viruses in Human Tracheal Epithelial Cells

	Oseltamivir	Zanamivir	Laninamivir	Peramivir
A/H1 pdm 2009	0.047 (0.032)**	0.009 (0.004)***	0.002 (0.001)***	0.001 (0.001)***
A/H1/Sendai/216/2009	1.03 (0.24)	0.035 (0.024)**	0.008 (0.003)***	0.53 (0.27)

A/H1 pdm 2009; influenza A/H1 pdm 2009 virus, A/H1/Sendai/216/2009; seasonal influenza A/H1N1 virus.
The cells were treated with one of four neuraminidase inhibitors (1 μM) from 30 min before infection to 5 days after infection, and the RNA was extracted from the cells. The results are expressed as the relative amount of RNA expression (ratio) compared to that of maximum influenza viral RNA on day 5 (120 hr) in the cells treated with vehicle and reported as the mean (SD) (n = 3). Significant differences compared to viral infection alone are indicated by $^{**}P < 0.01$ and $^{***}P < 0.001$.

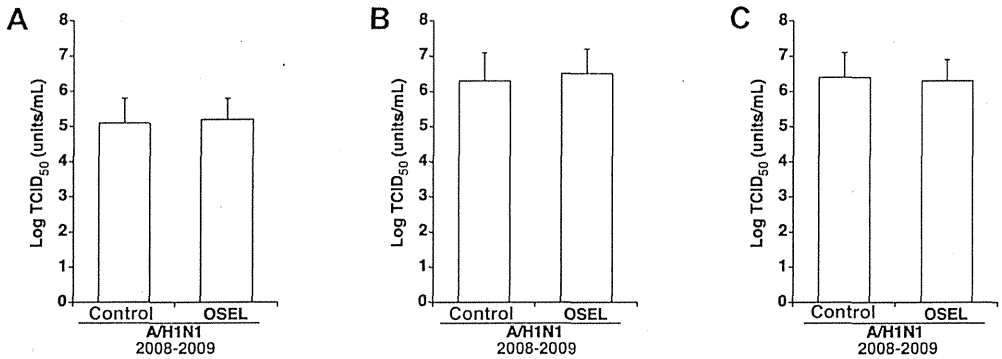

Fig. 2. **A**: Viral titers of the seasonal influenza A/H1N1 virus (A/H1N1 2008–2009) in the supernatants of primary cultures of human tracheal epithelial cells collected 9 days after infection. Treatment with oseltamivir (OSEL, 1 μM) or vehicle (control, 0.01% water) was initiated 3 days after infection and continued until the end of the experiments. The viral titers in the supernatants are expressed as $TCID_{50}$ units/ml. The results are expressed as the mean ± SD from three different tracheae. Treatment with oseltamivir did not reduce viral titers. **B,C**: Viral titers of the seasonal influenza A/H1N1 virus in supernatants collected 5 days after infection. Treatment with oseltamivir (OSEL, 1 μM) or vehicle (control) was initiated immediately after infection (B) or 30 min before infection (C) and continued until the end of the experiments. The viral titers in the supernatants are expressed as $TCID_{50}$ units/ml. The results are expressed as the mean ± SD from three different tracheae. Treatment with oseltamivir did not reduce viral titers.

Effects of Neuraminidase Inhibitors on Cytokine Production

Significant amounts of IL-6 were detected in the supernatants before viral infection, but the concentration of TNF-α in the supernatants prior to infection was below the detection level (0.55 pg/ml). Secretions of IL-6 and TNF-α increased after influenza A/H1 pdm 2009 viral infection (Table III). Maximum secretion of IL-6 and TNF-α was observed 5 days (data from days 1, 3, and 7 not shown) after infection. Significant amounts of TNF-α were detected in the supernatants after viral infection, but the concentration of TNF-α was much lower than that of IL-6 (Table III).

When the cells were pretreated with one of four types of inhibitors (oseltamivir, zanamivir, laninamivir, or peramivir, each at a concentration of 1 μM) from 30 min before, during, and after infection, the inhibitors reduced the concentrations of IL-6 and TNF-α in the supernatants 5 days after infection with the influenza A/H1 pdm 2009 virus (Table III).

Secretion of IL-6 and TNF-α also increased after oseltamivir-resistant seasonal influenza A/H1N1 viral infection (Table III). In contrast, treatment of the cells with oseltamivir (1 μM) from 30 min before, during, and after infection did not reduce the concentrations of IL-6 and TNF-α in the supernatants 5 days after infection (Table III). Zanamivir, laninamivir, and peramivir (1 μM) reduced the concentrations of IL-6 and TNF-α in the supernatants after infection with the seasonal influenza influenza A/H1N1 virus, but the magnitude of the inhibitory effects observed for peramivir (1 μM) was smaller than that observed for zanamivir and laninamivir (Table III).

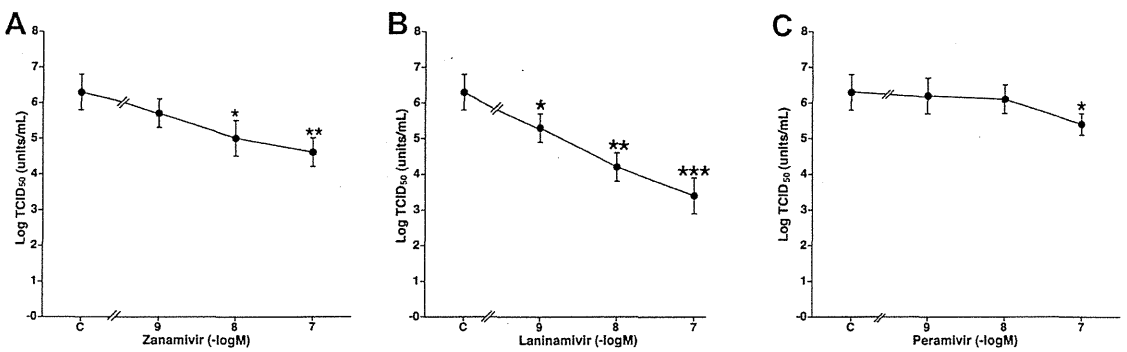

Fig. 3. **A–C**: Concentration-dependent effects of the neuraminidase inhibitors zanamivir (A), laninamivir (B), and peramivir (C) on the release of the seasonal influenza A/H1N1 virus in supernatants collected 5 days after infection. Primary cultures of human tracheal epithelial cells were treated with one of the neuraminidase inhibitors or vehicle (control, C; 0.01% water) from 30 min before infection until the end of the experiments. Changes in the virus concentration in the supernatants are expressed as $TCID_{50}$ units/ml. The results are expressed as the mean ± SD from three different tracheae. Significant differences compared to vehicle alone (C) are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

TABLE II. Neuraminidase Gene Mutation and Drug Sensitivity of Influenza Viruses

Virus strain	A/H1 pdm 2009	A/H1/Sendai/216/2009
Neuraminidase gene mutation (R/S)	None (S)	H275Y (R)
Drug susceptibility IC_{50} (nM, n = 3, mean ± SD)		
Oseltamivir	1.58 ± 0.19	568.61 ± 105.52
Zanamivir	0.53 ± 0.14	1.17 ± 0.25
Laninamivir	0.23 ± 0.04	0.34 ± 0.02
Peramivir	0.11 ± 0.03	56.72 ± 29.41

S, sensitive; R, resistant; IC_{50}, 50% inhibitory concentration; H275Y, a histidine-to-tyrosine amino acid substitution at position 275. A/H1 pdm 2009; influenza A/H1 pdm 2009 virus, A/H1/Sendai/216/2009; seasonal influenza A/H1N1 virus.

DISCUSSION

In the present study, oseltamivir, zanamivir, laninamivir, and peramivir reduced titers of the pandemic A/H1 2009 influenza (influenza A/H1 pdm 2009) virus in a concentration-dependent manner in supernatants of primary cultures of human tracheal epithelial cells, which retain the function of the original tissues [Yamaya et al., 1992]. In contrast, oseltamivir did not reduce viral titers and RNA of seasonal influenza A/H1N1 that was isolated during the 2008–2009 season. The IC_{50} of oseltamivir for the seasonal influenza A/H1N1 virus, as measured using a neuraminidase inhibition assay, was more than 300-fold higher than that observed for the influenza A/H1 pdm 2009 virus. The seasonal influenza A/H1N1 virus had an H275Y mutation, but the influenza A/H1 pdm 2009 virus did not have the same mutation. These findings suggest that the four types of neuraminidase inhibitors inhibit infection by the influenza A/H1 2009 pdm virus; in contrast, the seasonal influenza A/H1N1 virus is resistant to oseltamivir, as reported previously [Dharan et al., 2009; Saito et al., 2010].

The inhibitory effects of laninamivir against the neuraminidase activity of the influenza A/H1 pdm 2009 virus that were observed in this study are consistent with the effects on activity that were reported by Kubo et al. [2012]. However, the magnitude of the inhibitory effects of laninamivir on viral release into supernatants that was observed in this study was more potent than that observed in an animal model [Kubo et al., 2010]; this difference may be caused by differences between cell culture and animal models.

The influence of the neuraminidase inhibitors in the supernatants on the titration of viruses remains unclear. However, the effects of the neuraminidase inhibitors on viral titers in the supernatants were consistent with the effects of the same inhibitors on the IC_{50} values for the viruses and on the viral RNA in the cells. Therefore, the viral titers measured in the present study might be indicative of the true viral content in the supernatants.

Because patients receive neuraminidase inhibitors after influenza infection in clinical practice, we also examined the effects of neuraminidase inhibitors on influenza A/H1 pdm 2009 viral infection by treating the cells with neuraminidase inhibitors 3 days after influenza virus infection. Viral titers were reduced to less than 10% of the titers of cells treated with vehicle 2 days after the start of treatment. The relationship between the reduction of viral release from the cells and clinical symptoms is uncertain. However, the observed time course for the reduction of viral titers was similar to that observed for body temperature in patients, in which body temperature returned to less than 37.0°C over a 48-hr period after starting treatment with oseltamivir [Suzuki and Ichihara, 2008].

TABLE III. Effects of Neuraminidase Inhibitors on the Secretion of Cytokines Before and After Influenza Viral Infection

	IL-6 (pg/ml)		TNF-α (pg/ml)	
	A/H1 pdm 2009	A/H1/Sendai/216/2009	A/H1 pdm 2009	A/H1/Sendai/216/2009
Before infection	215 ± 14	218 ± 16	<0.55	<0.55
After infection	1,169 ± 87**	1,086 ± 83**	19.0 ± 1.2*	23.0 ± 1.8*
+ Oseltamivir	696 ± 57*+	1,022 ± 79**	<0.55+	22.7 ± 1.7*
+ Zanamivir	683 ± 55*+	728 ± 57*+	<0.55+	<0.55+
+ Laninamivir	428 ± 31*++	733 ± 55*+	<0.55+	<0.55+
+ Peramivir	441 ± 32*++	906 ± 78**+	<0.55+	15.8 ± 1.0*+

A/H1 pdm 2009; influenza A/H1 pdm 2009 virus, A/H1/Sendai/216/2009; seasonal influenza A/H1N1 virus, IL-6; interleukin-6, TNF; tumor necrosis factor.
The cells were treated with one of four neuraminidase inhibitors (1 μM) from 30 min before infection until 5 days after infection, when the supernatants were collected. The results are reported as the mean (SD) (n = 3). Significant differences compared to the values observed before infection are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$. Significant differences compared to viral infection alone are indicated by $^{+}P < 0.05$ and $^{++}P < 0.01$.

In the present study, influenza A/H1 pdm 2009 viral titers were reduced at concentrations of 1 nM or higher, regardless of the timing of neuraminidase inhibitor addition (i.e., before, immediately after or 3 days after infection). In human subjects, the mean plasma concentration of the active metabolite oseltamivir carbohydrate was approximately 560 nM 12 hr after intake [Abe et al., 2006] and the mean oseltamivir concentration in the sputum 2–4 hr after intake was 43 nM [Jullien et al., 2011]. Plasma concentrations of peramivir 18–24 hr after the end of infusion were 18–73 nM [Kohno et al., 2010]. Likewise, the mean concentration of zanamivir in the epithelial lining fluid 12 after inhalation was 2.7 μM [Shelton et al., 2011]. Furthermore, in mouse lungs, the concentration of laninamivir 12 hr after intranasal administration was more than 1.0 μM [Kubo et al., 2012]. The concentrations of the neuraminidase inhibitors reported in these studies demonstrate that the typical clinical concentrations of neuraminidase inhibitors are 1 nM or higher. Therefore, the results of the present study suggest that neuraminidase inhibitors can reduce influenza A/H1 pdm 2009 viral release into the supernatants of human tracheal epithelial cells at typical clinical concentrations.

Similar to the results related to viral shedding from patients reported by Ling et al. [2010], the influenza A/H1 pdm 2009 virus was detected in supernatants for at least 9 days in the current study. In contrast, Boivin et al. [2000] demonstrated that influenza virus titers in pharyngeal secretions were reduced to approximately zero by 5 days after the start of treatment with zanamivir. In the present study, influenza A/H1 pdm 2009 viral titers were detected 6 days after treatment with zanamivir, which is a longer duration than that reported by Boivin et al. [2000]. Although the reasons remain unclear, the absence of interferon in the supernatants, as reported previously [Terajima et al., 1997], might be partly associated with the long period of viral release from the cells in this study.

The IC_{50} values of the studied neuraminidase inhibitors (oseltamivir, zanamivir, laninamivir, and peramivir), as indicated by neuraminidase inhibition assays for the influenza A/H1 pdm 2009 virus and the oseltamivir-resistant seasonal influenza A/H1N1 virus, were consistent with those reported previously [Itoh et al., 2009; Okomo-Adhiambo et al., 2010; Suzuki et al., 2011; Pizzorno et al., 2012]. The magnitude of the effects of neuraminidase inhibitors (oseltamivir, zanamivir, and laninamivir) on viral release that was observed in the present study was also consistent with the 90% inhibitory concentration (IC_{90}) that was measured using MDCK cells by Itoh et al. [2009]. In contrast, 1 μM oseltamivir did not reduce the viral titers of the oseltamivir-resistant seasonal influenza A/H1N1 virus, which had the H275Y mutation [Saito et al., 2010; Suzuki et al., 2011]. The time course of the release of the influenza A/H1 pdm 2009 virus in the supernatants of primary cultures of human tracheal epithelial cells was similar to that observed in human bronchial epithelial cells by Itoh et al. [2009]. Furthermore, the magnitude of the IC_{50} values of the neuraminidase inhibitors, which was measured using a neuraminidase inhibition assay, for the seasonal influenza A/H1N1 virus was inversely associated with the magnitude of the inhibitory effects on the release of the virus in this study.

Yamashita et al. [2011] suggested that laninamivir, which is the active form of laninamivir octanoate [Kiso et al., 2010; Kubo et al., 2012], binds to neuraminidase in the Golgi apparatus and that the laninamivir–neuraminidase complex moves to the cell membrane and inhibits the budding of influenza virus particles. However, in the present study, laninamivir might have inhibited viral release from the cells by binding to the viral neuraminidase on the cell surface because the cells were treated with laninamivir by adding the drug to the culture medium.

The effects of neuraminidase inhibitors have been examined using various methods, including evaluating influenza neuraminidase activity [Bantia et al., 1998], survival rates of infected mice, and viral titers in the lungs of infected mice and in the supernatants of cultures of MDCK cells [Smee et al., 2001; Itoh et al., 2009; Yamashita et al., 2009]. A relationship between neuraminidase gene mutations and neuraminidase inhibitor resistance has also been reported [Kiso et al., 2004; Sheu et al., 2008; Saito et al., 2010]. In contrast, Boivin et al. [2000] and Sugaya and Ohashi [2010] measured influenza viral titers in pharyngeal throat swabs or secretions to evaluate the clinical effectiveness of neuraminidase inhibitors in naturally occurring influenza patients. These researchers also demonstrated that the reduction of influenza viral titers was associated with the reduction of the time of influenza symptoms in patients treated with zanamivir [Boivin et al., 2000] and laninamivir octanoate [Sugaya and Ohashi, 2010]. Because pharyngeal secretions contain influenza viruses that are replicated in airway cells, influenza viral titers in the supernatants of human tracheal epithelial cells may provide more relevant information about the clinical effectiveness of anti-influenza drugs than parameters measured using traditional methods, such as survival rates of infected mice and viral titers in the lungs of infected mice and in the supernatants of cultures of MDCK cells.

The release of influenza virus by human bronchial epithelial cells has been reported previously [Itoh et al., 2009], but the effects of anti-influenza drugs on the release of influenza viruses from primary cultures of human airway epithelial cells, which retain the functions of the original tissue [Yamaya et al., 1992], have not been well studied. In the present study, influenza viral stock was obtained by collecting the supernatants of primary cultures of human tracheal epithelial cells after infection with viruses isolated

from patient throat swabs. Furthermore, the effects of neuraminidase inhibitors on the release of influenza virus from human tracheal epithelial cells were examined to establish a new strategy for evaluating the effect of neuraminidase inhibitors on influenza viral replication in human airway epithelial cells. Four types of inhibitors reduced oseltamivir-sensitive influenza A/H1 pdm 2009 viral titers in supernatants and viral RNA in cells. In contrast, oseltamivir did not reduce viral titers and viral RNA of the oseltamivir-resistant seasonal influenza A/H1N1 virus. These findings suggest that accurate information related to the effectiveness of neuraminidase inhibitors on influenza virus replication in human airways can be acquired rapidly using the methods reported in this study. This information will be useful for physicians during the treatment of patients with influenza virus infection.

IL-6 and TNF-α are associated with airway inflammation during seasonal influenza viral infection [Hayden et al., 1998] and with the induction of apoptosis in cells [Ruwanpura et al., 2011; Gao et al., 2012]. The results of the present study demonstrated that four types of neuraminidase inhibitors can reduce the concentrations of IL-6 and TNF-α in supernatants after influenza A/H1 pdm 2009 viral infection, but treatment with oseltamivir does not reduce the concentrations of these cytokines after oseltamivir-resistant seasonal influenza A/H1N1 viral infection. Therefore, the inflammatory cytokines that are produced in tracheal epithelial cells may be related to airway damage induced by infection with the influenza A/H1 pdm 2009 virus and the seasonal influenza A/H1N1 virus. Furthermore, the methods established in the present study can be used to measure not only the viral titers but also the levels of inflammatory cytokines in the supernatants.

Affinity for and growth within the epithelium varies among different strains of influenza viruses. In fact, the maximum viral titers in the supernatants of cells infected with the influenza A/H1 2009 virus and the oseltamivir-resistant seasonal influenza A/H1N1 virus in this study were higher than the previously reported titers in the supernatants of cells infected with the seasonal influenza H3N2 virus [Yamaya et al., 2010]. The viral titers in the lungs of mice were also different among the strains [Kubo et al., 2010]. Kubo et al. [2010] determined the area under the curves (AUCs) of the time course changes of viral titers in the lungs of mice treated with zanamivir and laninamivir and calculated the ratio, which was compared to the AUC in mice treated with saline, to examine the effectiveness of the drugs against influenza virus infection. In methods that use cultured cells, the AUC ratio may indicate the magnitude of the effect of neuraminidase inhibitors on viral release. The IC_{90} of neuraminidase inhibitors for viral titers in the supernatants of the cells 24 hr after infection has also been reported [Itoh et al., 2009]. These parameters may standardize the observed differences in affinity for and growth in the epithelium among influenza strains.

In summary, the treatment of primary cultures of human tracheal epithelial cells with the neuraminidase inhibitors oseltamivir, zanamivir, laninamivir, and peramivir reduced the viral titers of influenza A/H1 pdm 2009 in cell supernatants in the present study. In contrast, oseltamivir did not reduce the titers of the oseltamivir-resistant seasonal influenza A/H1N1 virus. Because the virus stocks were prepared by infecting human tracheal epithelial cells using nasal swabs, these infection methods may provide accurate and rapid information related to the effects of neuraminidase inhibitors on influenza viruses that are isolated clinically from patients.

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ORIGINAL PAPER

Intracellular in vitro probe acylcarnitine assay for identifying deficiencies of carnitine transporter and carnitine palmitoyltransferase-1

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Abstract Mitochondrial fatty acid oxidation (FAO) disorders are caused by defects in one of the FAO enzymes that regulates cellular uptake of fatty acids and free carnitine. An in vitro probe acylcarnitine (IVP) assay using cultured cells and tandem mass spectrometry is a tool to diagnose enzyme defects linked to most FAO disorders. Extracellular acylcarnitine (AC) profiling detects carnitine palmitoyltransferase-2, carnitine acylcarnitine translocase, and other FAO deficiencies. However, the diagnosis of primary carnitine deficiency (PCD) or carnitine palmitoyltransferase-1 (CPT1) deficiency using the conventional IVP assay has been hampered by the presence of a large amount of free carnitine (C0), a key molecule deregulated by these deficiencies. In the present study, we developed a novel IVP assay for the diagnosis of PCD and CPT1 deficiency by analyzing intracellular ACs. When exogenous C0 was reduced, intracellular C0 and total AC in these deficiencies showed specific profiles clearly distinguishable from other FAO disorders and control cells. Also, the ratio of intracellular to extracellular C0 levels showed a significant difference in cells with these deficiencies compared with control. Hence, intracellular AC profiling using the IVP assay under reduced C0 conditions is a useful method for diagnosing PCD or CPT1 deficiency.

Keywords Fatty acid oxidation · Carnitine cycle disorder · Acylcarnitine profile · ESI-MS/MS

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Introduction

L-Carnitine plays an essential role in the transfer and activation of long-chain fatty acids across the outer and inner mitochondrial membranes during which it is acted upon by enzymes including carnitine transporter (OCTN2), carnitine palmitoyltransferase-1 (CPT1), carnitine palmitoyltransferase-2 (CPT2), and carnitine acylcarnitine translocase (CACT) (Fig. 1) [1, 2]. Carnitine penetrates into cells across the plasma membrane against a high concentration gradient of free carnitine with the aid of the plasma membrane OCTN2 protein encoded by the SLC22A5 gene [3]. Deficiency of OCTN2 causes primary carnitine deficiency (PCD, OMIM 212140), which is characterized by systemic carnitine deficiency in tissues and blood but in concord with increased excretion of free L-carnitine in the urine [4–6]. Clinical symptoms in patients with PCD such as cardiomyopathy,

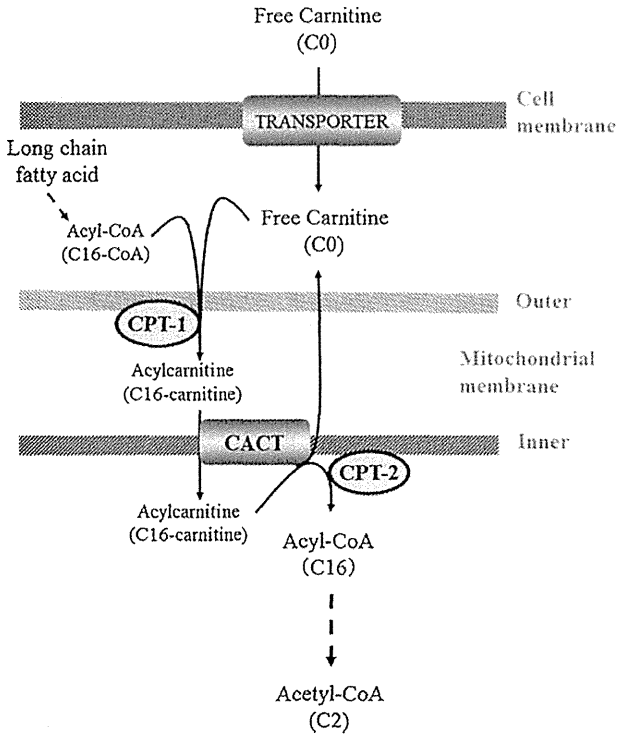

Fig. 1 Pathway for mitochondrial fatty acid beta-oxidation. Transporter: carnitine uptake transporter; *CPT-1*: carnitine palmitoyltransferase-1, *CACT*: carnitine acylcarnitine translocase, *CPT-2*: carnitine palmitoyltransferase-2. *Solid arrows* indicate single reactions; *dashed arrows* indicate multiple reactions or steps

encephalopathy, hepatomegaly, myopathy, hypoglycemia, and hyperammonemia, mainly result from low carnitine concentration in the tissues. On the other hand, secondary carnitine deficiency occurs in some conditions such as organic acidemias, renal dialysis, long-term medication (antiepileptic drugs or some antibiotics), and alimentary deficiency of L-carnitine [7–9].

It is necessary to make a differential diagnosis of PCD from the secondary carnitine deficiency or other false-positive cases, and diagnosis is confirmed by demonstrating reduced transport in skin fibroblasts from the patients. Until now, cluster-tray method using radioisotope-labeled substrate was used for the diagnosis of PCD [4, 10–12]. However, such a diagnostic method requires handling of radioactive substrates and focused only on diagnosis of PCD. Gene sequencing in SLC22A5 is one diagnostic method for PCD. However, it is molecularly heterogenous, and around 50 different mutations have been identified [6]. After acylcarnitine analysis using tandem MS analysis became available in the worldwide, blood acylcarnitine analysis was used as an initial method for diagnosis of FAO disorders and a detection of FAO disorders has been increased. However, it is necessary to confirm the diagnosis of the diseases with detailed analysis. The in vitro probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry (MS/MS) has been used to evaluate FAO capacity in the cultured cells and make a diagnosis of FAO disorders [13–15]. However, conventional IVP assay is not feasible to diagnose PCD or CPT1 deficiency, because excess amount of free carnitine is added to the experimental medium at the beginning. Estimation of free carnitine, which is the key marker for the above diseases, in experimental medium was nonsense for diagnosis of these disorders. We developed a novel functional assay for PCD and CPT1 deficiency using the IVP assay, with some modifications. This method uses different concentrations of exogenous free carnitine and measures intracellular as well as extracellular acylcarnitine (AC) levels, which overcomes the disadvantage of the conventional IVP assay in the diagnosis of carnitine cycle disorders.

Materials and methods

Materials

Hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), and palmitoylcarnitine (C16) were purchased from Sigma–Aldrich (St Louis, MO, USA). Methanol, acetonitrile, and formic acid were purchased from Wako (Osaka, Japan). As an internal standard, a labeled carnitine standard kit (NSK-B), which contains $^{2}[H]_{9}$-carnitine, $^{2}[H]_{3}$-acetylcarnitine, $^{2}[H]_{3}$-propionylcarnitine, $^{2}[H]_{3}$-butyrylcarnitine, $^{2}[H]_{9}$-isovalerylcarnitine, $^{2}[H]_{3}$-octanoylcarnitine, $^{2}[H]_{9}$-myristoylcarnitine, and $^{2}[H]_{3}$-palmitoylcarnitine, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Preparation of standard solutions of ACs

Standard solutions containing 1, 10, 25, and 50 μmol/L each of C6, C8, C10, and C16 were used to validate the recovery and determine linear concentration range of ACs after extraction by the Folch method [16]. The ACs were dissolved in methanol (99.8 %), and the prepared standard solution was analyzed directly and after extraction by the Folch method.

Subjects

Human skin fibroblasts from six healthy controls (volunteers) and seven patients with various carnitine cycle disorders—three each with PCD and CPT2 deficiency and one with CPT1 deficiency—were analyzed. In all cases, diagnoses were confirmed by mass spectrometric analyses (gas chromatography-mass spectrometry and MS/MS), enzyme assay, and protein or mutational analyses. Informed consent was obtained from the patients or their families. This study was approved by the Ethical Committee of the Shimane University School of Medicine.