Efficacy of Zanamivir against Avian Influenza A Viruses That Possess Genes Encoding H5N1 Internal Proteins and Are Pathogenic in Mammals

IRINA A. LENEVA,^{1,2} OLGA GOLOUBEVA,³ ROBERT J. FENTON,⁴ MARGARET TISDALE,⁴
AND ROBERT G. WEBSTER^{1,5*}

Department of Virology and Molecular Biology¹ and Department of Biostatistics and Epidemiology,³ St. Jude Children's Research Hospital, and Department of Pathology, University of Tennessee,⁵ Memphis, Tennessee 38105; Department of Chemotherapy of Infectious Diseases, Russian Chemical and Pharmaceutical Institute, Moscow, Russia²; and Glaxo Wellcome Research and Development, Stevenage, Hertfordshire, United Kingdom⁴

Received 31 July 2000/Returned for modification 25 October 2000/Accepted 24 January 2001

In 1997, an avian H5N1 influenza virus, A/Hong Kong/156/97 (A/HK/156/97), caused six deaths in Hong Kong, and in 1999, an avian H9N2 influenza virus infected two children in Hong Kong. These viruses and a third avian virus [A/Teal/HK/W312/97 (H6N1)] have six highly related genes encoding internal proteins. Additionally, A/Chicken/HK/G9/97 (H9N2) virus has PB1 and PB2 genes that are highly related to those of A/HK/156/97 (H5N1), A/Teal/HK/W312/97 (H6N1), and A/Quail/HK/G1/97 (H9N2) viruses. Because of their similarities with the H5N1 virus, these H6N1 and H9N2 viruses may have the potential for interspecies transmission. We demonstrate that these H6N1 and H9N2 viruses are pathogenic in mice but that their pathogenicities are less than that of A/HK/156/97 (H5N1). Unadapted virus replicated in lungs, but only A/HK/156/97 (H5N1) was found in the brain. After three passages (P₃) in mouse lungs, the pathogenicity of the viruses increased, with both A/Teal/HK/W312/97 (H6N1) (P₃) and A/Quail/HK/G1/97 (H9N2) (P₃) viruses being found in the brain. The neuraminidase inhibitor zanamivir inhibited viral replication in Madin-Darby canine kidney cells in virus yield assays (50% effective concentration, 8.5 to 14.0 μM) and inhibited viral neuraminidase activity (50% inhibitory concentration, 5 to 10 nM). Twice daily intranasal administration of zanamivir (50 and 100 mg/kg of body weight) completely protected infected mice from death. At a dose of 10 mg/kg, zanamivir completely protected mice from infection with H9N2 viruses and increased the mean survival day and the number of survivors infected with H6N1 and H5N1 viruses. Zanamivir, at all doses tested, significantly reduced the virus titers in the lungs and completely blocked the spread of virus to the brain. Thus, zanamivir is efficacious in treating avian influenza viruses that can be transmitted to mammals.

Since 1997, two avian H5N1 and H9N2 influenza viruses have been transmitted directly from birds to humans. A/Hong Kong/156/97 (A/HK/156/97) (H5N1) influenza virus caused 18 confirmed infections, with six deaths, in Hong Kong (3, 4, 5, 31). In 1998 and 1999, H9N2 influenza viruses infected five humans in China and two in Hong Kong (15, 19). The H5N1 and H9N2 influenza viruses that were transmitted to humans possess six genes encoding internal proteins that are closely related to each other (19) and to A/Quail/HK/G1/97 (H9N2), the likely source of the H9N2 viruses that were transmitted to children in Hong Kong. The increased prevalence of A/Quail/HK/G1/97 in poultry in China, together with serological evidence of infection in up to 60% of quail and up to 16% of quail shedding this virus in cages in Hong Kong poultry markets in 1999 to 2000 (10), increases the likelihood of transmission to mammals, including humans. Additionally, viruses of the H6N1 subtype that are similar to A/Teal/HK/W312/97, which possesses seven gene segments (PB2, PB1, PA, NA, NP, M, and NS), are highly related genetically to those of the H5N1 influenza viruses isolated from humans in 1997 and continue to circulate in domestic poultry (18). There is concern that these

H6N1 viruses containing H5N1 genomes will also be transmitted to humans.

Because the H9N2 and H6N1 influenza viruses containing H5N1 genes encoding internal proteins continue to circulate in poultry in southeast Asia (10), there is a potential risk of these avian influenza A viruses infecting and causing disease in mammals. It is important to determine the pathogenicities of these viruses, for they have the potential for interspecies transmission and hence the potential to cause epidemics and pandemics.

The most cost-effective approach in controlling epidemic and pandemic influenza is immunization; however, it takes at least 6 months to prepare a new vaccine, even under ideal conditions. When a vaccine is available, many people remain unvaccinated, despite widespread promotion of immunization. In the face of an emerging pandemic, antiviral drugs have enormous potential for preventing the deaths of infected persons and the further spread of disease. Mice are useful experimental animals for vaccine development, and previous studies have shown that H5N1 viruses are highly pathogenic in mice since they replicate without adaptation, cause systemic infection, and spread to the brain (7, 14, 20, 30).

Zanamivir (Relenza) is a neuraminidase (NA) inhibitor that was recently approved for use in humans in Europe, the United States, and Australia. It has significant antiviral activity in cell culture, in animals, and in humans (12, 16, 17, 33, 36, 39). How-

* Corresponding author. Mailing address: Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105-2794. Phone: (901) 495-3400. Fax: (901) 523-2622. E-mail: Robert.Webster@stjude.org.

ever, although it was shown that zanamivir failed to protect chickens from infection with highly pathogenic avian influenza viruses (22), zanamivir effectively protected mice from death when they were infected with A/HK/156/97 (14). It is therefore important to determine if zanamivir is efficacious against the H6N1 and H9N2 subtypes when it is presented on an H5N1 internal gene complex. To facilitate this aim, it was necessary to determine the pathogenicities of these emerging avian influenza viruses in mice for *in vivo* evaluation of zanamivir. These viruses included A/Teal/HK/W312/97 (H6N1), A/Quail/HK/G1/97 (H9N2), and A/Chicken/HK/G9/97 (H9N1). We compared the pathogenicities of these viruses in mice with that of A/HK/156/97 (H5N1) and determined the efficacies of zanamivir against these viruses both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Compounds. Zanamivir (4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuramic acid) was provided by Glaxo Wellcome Research and Development. The compound was dissolved in minimal essential medium (MEM) for *in vitro* experiments and in distilled water for *in vivo* experiments.

Cells and viruses. Madin-Darby canine kidney (MDCK) cells were grown in MEM containing 10% fetal bovine serum (FBS) and antibiotics. Influenza A/HK/156/97 (H5N1) virus was isolated from humans in Hong Kong in 1997 (5). A/Teal/HK/W312/97 (H6N1), A/Quail/HK/G1/97 (H9N2), and A/Chicken/HK/G9/97 (H9N2) viruses were isolated in Hong Kong from a teal duck, quail, and chicken, respectively. The viruses were serially passaged three times (P_3) in mouse lungs and were then propagated in embryonated chicken eggs. The resulting viruses were designated A/Teal/HK/W312/97 (H6N1) (P_3), A/Quail/HK/G1/97 (H9N2) (P_3), and A/Chicken/HK/G9/97 (H9N2) (P_3). All experiments were conducted at St. Jude Children's Research Hospital in a biosafety level 3 containment facility.

Mice. Female BALB/c mice were maintained under specific-pathogen-free conditions until they were used at 8 to 12 weeks of age. In these experiments, infected mice were killed 2, 4, and 7 days after infection and their lungs and brains were removed under sterile conditions. Organs were homogenized, suspended in a total volume of 1 ml of phosphate-buffered saline (PBS), serially diluted 10-fold, and assayed for virus infectivity in embryonated chicken eggs. Titers of infectious virus are presented as $\log_{10}$ 50% egg infectious doses (EID_{50}).

Antiviral assay. A modified enzyme-linked immunosorbent assay (ELISA) (2, 17) was used to determine the inhibitory effects of the anti-NA inhibitor zanamivir. This assay detected expression of viral NP protein in infected cells. Briefly, MDCK cells were seeded in 96-well culture plates at a density of 3,000 cells per well in MEM containing 10% FBS, 100 U of penicillin per ml, 100 μ g of streptomycin sulfate per ml, and 100 μ g of kanamycin sulfate per ml. Cells were incubated at 37°C with 5% CO_2 to reach 90% confluence. Cells were washed twice with serum-free MEM, and residual medium was removed. Each microtiter plate included uninfected control cultures, virus-infected control wells, and virus-infected cultures to which antiviral compounds were added. The cultures were overlaid with MEM containing 2.5 μ g of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin per ml and twice the concentration of the antiviral drug being studied (100 μ l). After incubation for 30 min at 37°C, 100 μ l of virus containing allantoic fluid (0.1 to 1.0 PFU/cell) was added to all wells, except the wells with the cell control. After incubation for 18 h at 37°C in a humidified atmosphere of 5% CO_2 , cells were fixed by addition of 100 μ l of cold acetone-PBS (80:20). The extent of viral replication was assessed by ELISA, as described previously (2).

The percent inhibition of virus replication by the antiviral compound was calculated after correction for the background (cell control) values with the following formula: percent inhibition = $100 \times (1 - OD_{450} \text{ of the treated sample} / OD_{450} \text{ of the virus control sample})$, where OD_{450} is the optical density at 450 nm. The concentrations of the compound that effectively inhibited virus replication by 50% (EC_{50}) were determined by plotting inhibition of virus replication as a function of compound concentration.

An MDCK plaque assay was used to study the sensitivity of the virus isolated from mouse lungs at the end of therapy. MDCK monolayers (six-well plates) were infected with viruses from lungs of mice treated or not treated with zanamivir and then overlaid with agar containing the antiviral compound at concen-

trations ranging from 0.03 to 10 μ M. After 4 days of incubation, the agar was removed and the plaques were visualized by crystal violet staining.

NA inhibition assay. NA activity was measured by the colorimetric assay (1), with fetuin as a substrate. The inhibitory effect of zanamivir on viral NAs was determined by assaying for enzyme activity in the presence of different concentrations of compound. Viruses used in NA inhibition tests were diluted in PBS to give a standard level of enzyme activity (0.5 OD_{540} unit). Tenfold dilutions of compound ranging from 0.0001 to 100 nM were incubated with viruses for 30 min at room temperature and then with fetuin overnight. Assays were performed in triplicate. The drug concentrations of zanamivir required to reduce enzyme activity to 50% (IC_{50}) were determined against all tested viruses.

Infection and drug administration in mice. Mice were anesthetized by inhalation of metofane and were inoculated by intranasal administration of virus in a volume of 100 μ l. Zanamivir was administered intranasally twice daily for 5 days, with the first dose being given 4 h before the mice were exposed to virus (14). Groups of 5 or 10 mice were used for each dose. For all mice, the changes in weight and the number of mice that died were recorded daily. The mean survival day (MSD), i.e., the mean number of days mice survived, was calculated by the following formula: $MSD = \sum[f(d - 1)]/n$, where f is the number of mice recorded on day d (the number of survivors on day 16 was included in f for that day) and n is the number of mice in a group (8).

Statistical analysis. The effects of different doses of drug in mice infected with different viruses on survival rate were evaluated using logistic regression and Fisher's exact test. Differences in lung and brain virus titers were compared with control values using a multiple-comparison method (Dunnett's two-tailed t test) available with the Analysis of Variance procedure.

RESULTS

Characterization of influenza viruses of avian origin in the mouse model. Mice are not a natural host for influenza viruses; unadapted influenza viruses usually replicate in the lungs of mice and cause asymptomatic infection of the respiratory tract (32, 37). In contrast, some unadapted H5N1 isolates, including A/HK/156/97 (H5N1), are highly pathogenic and neurotropic in mice (6, 7, 14, 20, 30). A/Quail/HK/G1/97 (H9N2) and A/Teal/HK/W312/97 (H6N1) possess genes encoding internal proteins that are highly related genetically to those of A/HK/156/97 (H5N1), whereas A/Chicken/HK/G9/97 (H9N2) has two genes encoding internal proteins, PB1 and PB2, that are highly related genetically to those of A/HK/156/97 (H5N1) (9). Therefore, we wished to establish the pathogenicities of A/Chicken/HK/G9/97 (H9N2), A/Quail/HK/G1/97 (H9N2), and A/Teal/HK/W312/97 (H6N1) for mice, in comparison with that of A/HK/156/97 (H5N1).

Titration of the H6N1 and H9N2 viruses in eggs showed that both viruses replicated to high titers in chicken eggs; the EID_{50} ranged from $10^{8.0}$ to $10^{9.0}$ and were similar for the viruses studied (Table 1). Our experiments confirmed that A/HK/156/97 (H5N1) virus is highly pathogenic to mice. All mice infected with virus diluted 10^{-1} to 10^{-6} lost up to 10 to 15% of their body weight and died by day 6 after infection. The dose that was lethal for 50% of the mice (MLD_{50}) infected with A/HK/156/97 (H5N1) was estimated to be 7.7 $\log_{10}$ EID_{50} and contained 1.16 EID_{50} . All mice showed signs of infection, including huddling and ruffled fur, and the mice in the group infected with 1 MLD_{50} lost 15% of their body weight.

A/Teal/HK/W312/97 (H6N1), which had not been serially passaged in mouse lungs, was less pathogenic, but all mice inoculated with the undiluted virus died of infection. The MLD_{50} for this virus was estimated to be 1.0 $\log_{10}$ EID_{50} and contained 8.5 EID_{50} . All mice infected with 1 to 10 MLD_{50} s of A/Teal/HK/W312/97 (H6N1) lost 20 to 24% of their body weight. After three passages of A/Teal/HK/W312/97 (H6N1) virus in the lungs of mice, the pathogenicity increased; the

TABLE 1. Replication of avian influenza viruses containing the internal genes of 1997 H5N1 viruses in chicken embryos and mice

Virus (passage)	Dilution	Virus titer			No. of mice that died/ total no. of mice	Mean % of wt lost on day 3 after infection $\pm$ SD
		Eggs (log EID ₅₀)	Mice (log MLD ₅₀)	MLD ₅₀ (EID ₅₀) ^a		
A/HK/156/97 (P ₀)		9.0	7.7	1.16		
	10 ⁻⁶				5/5	-15.4 $\pm$ 1.0
	10 ⁻⁷				4/5	-16.2 $\pm$ 0.9
	10 ⁻⁸				2/5	-10.4 $\pm$ 0.8
	10 ⁻⁹				0/5	-5.3 $\pm$ 0.8
A/Teal/HK/W312/97 (P ₀)		8.5	1.0	8.5		
	Undiluted				5/5	-24.1 $\pm$ 2.0
	10 ⁻¹				2/5	-20.4 $\pm$ 1.7
	10 ⁻²				0/5	-8.0 $\pm$ 1.1
A/Teal/HK/W312/97 (P ₃) ^b		8.2	3.5	2.3		
	10 ⁻¹				5/5	-25.3 $\pm$ 0.7
	10 ⁻²				5/5	-25.5 $\pm$ 1.0
	10 ⁻³				4/1	-24.3 $\pm$ 2.0
	10 ⁻⁴				1/4	-10.1 $\pm$ 1.1
A/Quail/HK/G1/97 (P ₀)		8.0	0.4	20.0		
	Undiluted				2/5	-10.0 $\pm$ 0.8
	10 ⁻¹				0/5	-8.1 $\pm$ 0.5
A/Quail/HK/G1/97 (P ₃) ^b		8.0	2.5	3.2		
	Undiluted				5/5	-20.3 $\pm$ 0.8
	10 ⁻¹				5/5	-20.5 $\pm$ 1.5
	10 ⁻²				5/5	-18.1 $\pm$ 0.9
	10 ⁻³				0/5	-16.6 $\pm$ 2.0
A/Chicken/HK/G9/97 (P ₀)		8.0	1.0	8		
	Undiluted				5/5	-24.4 $\pm$ 0.7
	10 ⁻¹				2/5	-20.0 $\pm$ 2.1
	10 ⁻²				1/5	-25.5 $\pm$ 0.9
	10 ⁻³				0/5	-18.0 $\pm$ 1.2
A/Chicken/HK/G9/97 (P ₃) ^b		8.2	2.3	3.2		
	10 ⁻¹				5/5	-22.2 $\pm$ 0.9
	10 ⁻²				3/2	-14.4 $\pm$ 0.5
	10 ⁻³				1/4	-12.8 $\pm$ 1.0

^a MLD₅₀ (EID₅₀), number of infectious virus units in 1 MLD₅₀.

^b A/Quail/HK/G1/97 (P₃), A/Chicken/HK/G9/97 (P₃), and A/Teal/HK/W312/97 (P₃) viruses were passaged in mouse lungs three times and then propagated in embryonated chicken eggs.

MLD₅₀ increased by almost 1,000-fold, and 1 MLD₅₀ contained 2.3 EID₅₀.

A/Quail/HK/G1/97 (H9N2) virus was the least pathogenic virus of those studied, and when undiluted, the virus did not kill all mice in the group. When this virus was serially subcultured three times in mouse lungs, the MLD₅₀ increased from 0.4 to 2.5 log₁₀ MLD₅₀, and the amount of infectious virus units in 1 MLD₅₀ decreased from 20.0 to 3.2 EID₅₀. Mice infected with A/Quail/HK/G1/97 (H9N2) (P₃) virus that was diluted 10-fold and 100-fold lost approximately 18 to 20% of their body weight, whereas mice infected with undiluted and unadapted A/Quail/HK/G1/97 (H9N2) virus lost only 10%.

When mice were infected with undiluted and unadapted A/Chicken/HK/G9/97 (H9N2), all mice died. The pathogenicity of the virus increased after three passages in mice; the MLD₅₀ increased from 1.0 to 2.3 log₁₀ MLD₅₀. The MLD₅₀ of the unadapted virus contained 8 EID₅₀, and 1 MLD₅₀ of the serially passaged virus contained 3.2 EID₅₀.

To further examine the pathogenicity of these avian viruses,

we compared their levels of growth in the lungs and brains of infected mice. Mice were infected with 5 MLD₅₀s of each virus, and three mice in each group were killed 1, 2, 3, 4, 5, and 7 days after infection. The titers of virus in the lungs and brains were determined (Table 2).

On day 1, mice infected with A/HK/156/97 (H5N1) virus had high titers in the lungs (6.0 to 6.5 log₁₀ EID₅₀) and reached a peak on day 3 (log₁₀ 8.5). High titers continued to be present through to day 5 (7.5 log₁₀ EID₅₀). A/HK/156/97 (H5N1) virus (titer, log₁₀ 2.0) was found in the brain on days 3 and 5 after infection. Similar results had previously been obtained by Gubareva et al. (14), Gao et al. (7), and Lu et al. (20). Although the viral titer in the brain did not reach the level of that in the lungs, the presence of A/HK/156/97 (H5N1) virus in the brain confirms that this virus can spread systemically in mice.

A/Teal/HK/W312/97 (H6N1) influenza virus replicated in mouse lungs to high titers, similar to those of A/HK/156/97 (H5N1) virus, but was not detected in brains of mice on any day after infection. After three passages in mouse lungs,

TABLE 2. Replication of avian influenza viruses (containing internal genes of 1997 H5N1 viruses) in mice

Virus ^b	Virus titer (log ₁₀ EID ₅₀ /ml ± SD) ^a					
	Lungs			Brain		
	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
A/HK/156/97 (H5N1) (P ₀)	6.0 ± 1.0	8.5 ± 0.7	7.5 ± 0.5	—	2.0 ± 0.8	2.0 ± 0.0
A/Teal/HK/W312/97 (H6N1) (P ₀)	6.5 ± 0.5	8.5 ± 0.9	7.5 ± 0.5	—	—	—
A/Teal/HK/W312/97 (H6N1) (P ₃)	NT	8.0 ± 1.0	NT	NT	2.5 ± 0.0	2.0 ± 0.0
A/Quail/HK/G1/97 (H9N2) (P ₀)	NT	5.2 ± 0.7	NT	NT	—	NT
A/Quail/HK/G1/97 (H9N2) (P ₃)	6.0 ± 0.0	9.0 ± 0.0	7.2 ± 0.3	—	2.0 ± 0.5	2.0 ± 0.0
A/Chicken/HK/G9/97 (H9N2) (P ₀)	4.5 ± 0.5	4.8 ± 0.9	2.5 ± 0.3	—	—	—
A/Chicken/HK/G9/97 (H9N2) (P ₃)	NT	7.4 ± 0.4	NT	NT	—	—

^a Three mice in each group were sacrificed at the indicated times, and their lungs were homogenized separately and treated as described in Materials and Methods. —, virus was not detected; NT, not tested.

^b A/Teal/HK/W312/97 (H6N1) (P₀), A/Quail/HK/G1/97 (H9N2) (P₀), and A/Chicken/HK/G9/97 (H9N2) (P₀) are the original viruses. A/Teal/HK/W312/97 (H6N1) (P₃), A/Quail/HK/G1/97 (H9N2) (P₃), and A/Chicken/HK/G9/97 (H9N2) (P₃) are original viruses that were passaged three times in mouse lungs.

A/Teal/HK/W312/97 (H6N1) (P₃) continued to replicate in the lungs to high titers and virus was found in the brains of mice 3 days after infection.

Titers of A/Quail/HK/G1/97 (H9N2) virus in the lungs were lower than those of A/HK/156/97 (H5N1) and A/Teal/HK/W312/97 (H6N1) viruses, but after three passages in the lungs of mice, the titer increased and was comparable to those found in the lungs of mice infected with A/HK/156/97 (H5N1) and A/Teal/HK/W312/97 (H6N1) viruses. Although we did not detect unadapted A/Quail/HK/G1/97 virus in the brains of mice, this virus, after three passages in mice, replicated in the brain to a titer of 2.0 log₁₀ EID₅₀ and continued to replicate at the same level on day 5 after infection.

The unadapted A/Chicken/HK/G9/97 (H9N2) virus replicated in the lungs of mice to titers of 4.5 log₁₀ EID₅₀ on the first day after infection and to 4.8 log₁₀ EID₅₀ by the third day. On day 5, the titer had decreased to 2.5 log₁₀ EID₅₀. After three passages in mice, this virus replicated to high titers in the lungs of mice. We did not detect virus in the brains of mice infected with either the unadapted or the adapted (serially passaged in mouse lungs) A/Chicken/HK/G9/97 (H9N2) virus.

Our study of the pathogenicity of the avian H6N1 and H9N2 influenza viruses possessing H5N1 genes showed that A/Teal/HK/W312/97 (H6N1), A/Quail/HK/G1/97 (H9N2), and A/Chicken/HK/G9/97 (H5N1) viruses are clearly less pathogenic in mice than is the A/HK/156/97 virus. Viruses passaged in mouse lungs had increased pathogenicity, but this pathogenicity did not reach the level seen for the A/HK/156/97 virus.

In vitro activity of zanamivir against influenza viruses of avian origin. Because the H6N1 and H9N2 viruses contain gene segments encoding internal proteins similar to those of A/HK/156/97 (H5N1), replicate in mice, and are in some cases neurotropic, these viruses have the potential to spread to humans. We therefore wished to determine the efficacies of zanamivir against these viruses. Zanamivir inhibited the replication of A/Quail/HK/G1/97 (H9N2), A/Chicken/HK/G9/97 (H9N2), and A/Teal/HK/W312/97 (H6N1) in MDCK cells, and the level of inhibition increased with increasing concentration (Fig. 1). The mean EC₅₀ were similar for all of the viruses tested and ranged from 8.5 to 14.0 μM (Table 3).

Because the target of zanamivir is the active site of NA, we estimated the efficacies of zanamivir against the H6N1 and H9N2 viruses in NA inhibition tests. The NA inhibition assays demonstrated that zanamivir efficiently inhibited the enzyme activities of the H6N1 and H9N2 viruses (Table 3). The IC₅₀ were 5.0 nM against A/HK/156/97 (H5N1), 7 ± 1 nM against A/Quail/HK/G1/97 (H9N2), 10 ± 2 nM against A/CK/HK/G9/97 (H9N2), and 7.5 ± 2.5 nM against A/Teal/HK/W312/97. Thus, zanamivir inhibited both NA activity and virus replica-

FIG. 1. Inhibition of H5N1, H6N1, and H9N2 influenza viruses in MDCK cells by zanamivir. MDCK cells were grown in 96-well plates and infected with viruses in the presence of zanamivir as described in Materials and Methods. The levels of NP protein expression were measured by ELISA. Each point is the average of results from four replicate wells ± standard deviations (SD) calculated for three independent experiments.

TABLE 3. In vitro effect of zanamivir on replication of influenza viruses of avian origin in MDCK cells and inhibition of their NA activities

Virus	Mean IC ₅₀ ± SD (nM) ^a	Mean EC ₅₀ ± SD (μM) ^b
A/HK/156/97 (H5N1)	5 ± 0	10 ± 2
A/Teal/HK/W312/97 (P ₀) (H6N1)	7.5 ± 2.5	8.5 ± 1.5
A/Quail/HK/G1/97 (P ₀) (H9N2)	7 ± 1	12 ± 4
A/Quail/HK/G1/97 (P ₃) (H9N2)	7 ± 0	10 ± 3
A/Chicken/HK/G9/97 (P ₀) (H9N2)	10 ± 2	14 ± 5

^a Determined in NA inhibition tests as described in Materials and Methods.^b Determined by cell ELISA using monoclonal antibodies to the NP protein of influenza A virus as described in Materials and Methods. Values are averages of results from three independent experiments.

tion in MDCK cells for each virus, with the levels of inhibition for the different viruses being comparable (Table 3).

Efficacies of zanamivir against A/Quail/HK/G1/97 (H9N2), A/Chicken/HK/G9/97 (H9N2), A/Teal/HK/W312/97 (H6N1), and A/HK/156/97 (H5N1) virus infection in mice. We used weight changes and death caused by H6N1 and H9N2 viruses in mice to determine the efficacy of zanamivir administered intranasally (Table 4). At doses of 50 mg/kg, zanamivir completely protected mice infected with A/HK/156/97 (H5N1) from death, and at doses of 10 mg/kg, zanamivir increased the number of survivors and mean survival time to 10.6 days, compared to 6.0 days for the control group of mice. When doses of 1 mg/kg were used, weight loss and mean survival time for the treatment group did not differ from those of the control groups.

Similar results were obtained when we studied the effect of zanamivir on A/Teal/HK/W312/97 infection in mice. Zanamivir at doses of 1 mg/kg did not prevent the weight loss and

death of mice infected with A/Teal/HK/W312/97, but doses of 50 mg/kg provided complete protection from weight loss and death. When compared against values for the control group, the number of survivors and the MSD of mice treated with the 10-mg/kg dose increased.

Because undiluted A/Quail/HK/G1/97 (H9N2) virus was not 100% lethal in mice, we tested the efficacy of zanamivir against virus that had been serially passaged in the lungs of mice three times. At doses of 10, 50, and 100 mg/kg, zanamivir administered intranasally prevented the deaths of mice infected with A/Quail/HK/G1/97 (P₃) virus. A dose of 1 mg of zanamivir per kg did not protect mice from weight loss and death.

Zanamivir administered intranasally at doses of 10, 50, and 100 mg/kg fully protected mice infected with A/Chicken/HK/G9/97 (H9N2) influenza virus from death. Nevertheless, mice infected with A/Chicken/HK/G9/97 (H9N2) and treated with 10 mg of zanamivir per kg lost more weight on days 3 and 7 after infection than did those treated with 50 or 100 mg/kg. When infected mice were treated with 1 mg of zanamivir per kg, the number of survivors and MSD increased compared with those of mice in the control group.

Our findings show that A/Quail/HK/G1/97 (H9N2) and A/Chicken/HK/G9/97 (H9N2) viruses appear to be more sensitive to zanamivir than A/Teal/HK/W312/97 (H6N1) and A/HK/156/97 (H5N1) viruses. Because we used 5 MLD₅₀s of virus in each challenge dose, the difference in antiviral effect was not due to differences in virus challenge doses.

Influence of challenge dose on the efficacy of zanamivir in mice infected with the A/HK/156/97 (H5N1) and A/Quail/HK/G1/97 (H9N2) viruses. Because differences among the sensitivities of influenza viruses to zanamivir cannot be due to

TABLE 4. Effect of intranasally administered zanamivir on avian influenza A viruses in mice

Virus ^a	Treatment	Dose (mg/kg/day)	No. of mice that survived/ total no. of mice	% Increase or decrease in body weight ± SD on postinfection day:				MSD ± SD
				3	7	11	15	
A/HK/156/97 (H5N1)	Zanamivir	1	0/5 ^b	-6.0 ± 0.5	-13 ± 0.9	— ^c	—	6.0 ± 0.0
		10	2/5 ^b	-2.1 ± 0.8	-7.1 ± 1.0	+7.0 ± 0.3	+17 ± 1.9	10.2 ± 1.2
		50	5/5 ^c	0.3 ± 0.1	-6.0 ± 1.2	+4.8 ± 0.2	+13 ± 1.2	>16
	PBS		0/4	-7.6 ± 0.2	-15	—	—	6.0 ± 0.0
A/Teal/HK/W312/97 (H6N1)	Zanamivir	1	0/5 ^b	-8.2 ± 0.7	—	—	—	4.0 ± 0.0
		10	2/5 ^b	0.4 ± 0.0	-2.2 ± 0.1	+1.3 ± 0.1	+2.1 ± 0.1	8.4 ± 1.6
		50	5/5 ^c	0.2 ± 0.0	-1.1 ± 0.2	+2.2 ± 0.2	+2.3 ± 0.5	>16
	PBS		0/5	-11.3 ± 0.9	—	—	—	4.0 ± 0.0
A/Quail/HK/G1/97 (P ₃) (H9N2) ^d	Zanamivir	1	0/10 ^b	-15.3 ± 0.9	—	—	—	4.9 ± 0.6
		10	10/10 ^c	-10.6 ± 1.1	-15.0 ± 1.9	-10.2 ± 0.7	+1.7 ± 0.1	>16
		50	10/10 ^c	-7.4 ± 1.0	-10.0 ± 0.2	-10.3 ± 0.3	+2.3 ± 0.5	>16
		100	10/10 ^c	-6.0 ± 0.2	-5.1 ± 0.2	-3.1 ± 0.0	+2.0 ± 0.1	>16
	PBS		0/10	-18.3 ± 0.2	—	—	—	4.0 ± 1.0
A/Chicken/HK/G9/97 (H9N2)	Zanamivir	1	2/10 ^b	-12.2 ± 0.3	-15.3 ± 1.0	-3.3 ± 0.1	+1.4 ± 0.0	7.8 ± 1.2
		10	10/10 ^c	-11.3 ± 0.2	-12.2 ± 0.9	-2.1 ± 0.0	+2.7 ± 0.1	>16
		50	9/9 ^c	-4.1 ± 0.1	-5.5 ± 0.0	+1.3 ± 0.0	+2.0 ± 0.1	>16
		100	10/10 ^c	-5.5 ± 0.0	-6.1 ± 0.2	+2.0 ± 0.7	+2.0 ± 0.6	>16
	PBS		0/10	-15.4 ± 0.5	—	—	—	3.6 ± 1.4

^a Five MLD₅₀s of each virus were used.^b The difference in survival rate between control and treated groups was not statistically significant at the 0.05 level.^c *P* < 0.001 for control (PBS) versus treated groups challenged with the same virus.^d A/Quail/HK/G1/97 (H9N2) passaged in mouse lungs three times was used in this experiment.^e —, the mice had died.

TABLE 5. Effects of zanamivir on high and low challenge doses of A/HK/156/97 (H5N1) and A/Quail/HK/G1/97 (H9N2) in mice

Virus	Treatment	Dosage (mg/kg/day)	High virus challenge (100 MLD ₅₀ s)		Low virus challenge (5 MLD ₅₀ s)	
			No. of mice that survived/total no. of mice	MSD $\pm$ SD	No. of mice that survived/total no. of mice	MSD $\pm$ SD
A/Quail/HK/G1/97 (H9N2) (P ₃) ^d	Zanamivir	0.05	0/5	3.6 $\pm$ 1.0	Not tested	Not tested
		0.1	0/5	3.8 $\pm$ 0.4	0/10	4.9 $\pm$ 0.6
		1	0/5	4.2 $\pm$ 0.4	0/10	5.5 $\pm$ 0.5
		10	2/5 ^a	9.3 $\pm$ 0.6	10/10 ^c	>16
		50	5/5 ^c	>16	10/10 ^c	>16
	PBS		0/5	3.0 $\pm$ 0.0	0/10	4.0 $\pm$ 0.0
A/HK/156/97 (H5N1)	Zanamivir	1	0/10	6.0 $\pm$ 0.8	0/5	6.0 $\pm$ 0.0
		10	0/10	6.0 $\pm$ 1.0	2/5 ^a	10.3 $\pm$ 0.6
		50	3/10	8.6 $\pm$ 0.4	5/6 ^b	>16
		100	8/10 ^c	13.5 $\pm$ 0.5	Not tested	>16
			0/10	5.5 $\pm$ 1.0	0/4	6.0 $\pm$ 0.0
	PBS		0/10			

^a The difference in survival rate between control and treated mice was not statistically significant at the 0.05 level.

^b $P < 0.05$ for comparison between control (PBS) and treated groups challenged with the same virus.

^c $P < 0.001$ for comparison between control (PBS) and treated groups challenged with the same virus.

^d A/Quail/HK/G1/97 was passaged three times in mouse lungs and used in experiments.

differences in the low doses of virus administered, we next wished to determine whether high challenge doses would also reveal differences in efficacy. It had been reported previously that zanamivir failed to protect chickens against high doses of a systemic influenza virus (12, 22), so we wished to determine whether this finding also applied in the mouse model system. Groups of mice were infected with low (5 MLD₅₀s) and high (100 MLD₅₀s) doses of A/HK/156/97 (H5N1) and A/Quail/HK/G1/97 (P₃) (H9N2) influenza viruses and then treated with different doses of zanamivir (Table 5). When mice were infected with low challenge doses of A/Quail/HK/G1/97 (P₃) (H9N2), zanamivir at 10 mg/kg completely protected mice from death, whereas a dose of 50 mg of zanamivir per kg was required to protect all mice infected with a low challenge dose of A/HK/156/97 (H5N1). At high challenge doses of A/Quail/HK/G1/97 (P₃) (H9N2), 50 mg/kg was needed to prevent the deaths of all mice. At a high challenge of A/HK/156/97 (H5N1) virus, 100 mg of zanamivir per kg was needed to protect 8 of 10 mice from death. Thus, the differences between the sensitivities of influenza viruses to zanamivir are also found when mice are challenged with high doses of virus. In the mouse, zanamivir was effective against a high challenge dose of a systemic influenza virus, albeit at higher doses.

Effect of zanamivir administered intranasally on lung and brain infection in mice. The above-described experiments demonstrated the efficacy of zanamivir in preventing the weight loss and death of mice infected with H5N1, H6N1, and H9N2 viruses. We also evaluated the extent of reduction of virus titers in the lungs of mice and showed that zanamivir significantly reduced the levels of virus for each of the four influenza viruses tested (Fig. 2). Doses of 1 mg/kg significantly reduced the level of A/Quail/HK/G1/97 (H9N2) virus in the lungs of mice on day 4 ($P < 0.01$), though this dose of inhibitor was less effective on the other viruses tested (Fig. 2). At higher doses (10 and 50 mg/kg), zanamivir significantly reduced titers, but the only virus for which the levels were reduced to undetectable levels was A/Chicken/HK/G9/97 (H9N2), the virus that grew to the lowest titer in mouse lungs.

On day 7 after treatment with 50 mg of zanamivir per kg, the

residual virus in the lungs of mice infected with A/HK/156/97 (H5N1), A/Teal/HK/W312/97 (H6N1), and A/Quail/HK/G1/97 (H9N2) (P₃) was examined for sensitivity to zanamivir by both ELISA and NA inhibition assay. Each of the residual viruses examined was as sensitive to zanamivir as the original virus (results not shown); these findings indicate that the viruses were not resistant to zanamivir.

Experiments were also done to determine the presence of virus in the brains of mice infected with A/HK/156/97 (H5N1) and A/Quail/HK/G1/97 (H9N2) (P₃) and treated with different doses of zanamivir. Virus was not detected in brain after treatment with 1, 10, or 50 mg/kg on any day after infection (data not shown).

DISCUSSION

Since 1997, two avian influenza viruses have been transmitted directly from birds to humans. H5N1 influenza virus caused 18 confirmed infections, with six deaths, in Hong Kong (3, 4, 5, 31). An influenza virus genetically highly related to A/Quail/HK/G1/97 (H9N2) virus infected two persons in Hong Kong (19, 26) and five persons in China (15). Examination of the consensus amino acid sequences of the internal virion proteins of A/HK/156/97 (H5N1) revealed that the PB2, PA, NP, and M2 proteins possess amino acids previously found in human strains (40). It has been proposed that the presence of human-specific amino acids in these H5N1 viruses permitted transmission of avian influenza viruses to humans. Because A/Quail/HK/G1/97 (H9N2), A/Chicken/HK/G9/97 (H9N2), and A/Teal/HK/W312/97 (H6N1) contain gene segments highly related genetically to those of A/HK/156/97 (H5N1), they have the potential to be transmitted to mammals.

Our findings established that the H9N2 and H6N1 viruses in this study can replicate and cause signs of disease in mice. It has already been shown that A/HK/156/97 (H5N1) virus causes lethal neurotropic infections of mice without adaptation (7, 14, 20). The present study established that A/Quail/HK/G1/97 (H9N2) and A/Teal/HK/W312/97 (H6N1) are much less pathogenic than A/HK/156/97 (H5N1) and that adaptation of

FIG. 2. Effects of different doses of zanamivir on titers of H5N1, H6N1, and H9N2 viruses from lungs of infected mice. Groups of mice were infected with viruses and treated with different doses of zanamivir or PBS (control group). Three mice from each dose group were sacrificed 2, 4, and 7 days after infection, and lungs were assayed separately for virus infectivity in eggs. *, the difference between values for control and drug-treated groups was not statistically significant; **, $P < 0.05$ compared to the value for the control group; ***, $P < 0.01$ compared to the value for the control group. In these experiments we used A/Quail/HK/G1/97 that was passaged in mouse lungs three times.

the viruses in mice was necessary to increase their pathogenicities. Experiments with A/Chicken/HK/G9/97 (H9N2) gave similar results, in that the virus was much less pathogenic than A/HK/156/97 (H5N1), and even after three passages in mice, virus was not detectable in the brains of infected mice. Thus, the H9N2 and H6N1 influenza viruses possessing six or seven gene segments similar to those of the H5N1 Hong Kong viruses contain the necessary group of genes for systemic spread in mice, but the absence of the H5 hemagglutinin (HA) gene may reduce the level of pathogenicity. The failure of A/Chicken/HK/G9/97 (H9N2) to infect the brain indicates that the PB1 and PB2 genes are insufficient for systemic spread to the brain. It is well established that host-range transmission and pathogenicity are polygenic traits (27, 29). Thus, the A/HK/156/97 (H5N1) virus has a better group of genes for both efficient systemic spread and the killing of mice than do the A/Quail/HK/G1/97 (H9N2) and A/Teal/HK/W312/97 (H6N1) viruses. However, the groups of genes in the latter two viruses are clearly sufficient for systemic spread in mice. These groups of genes are apparently associated with interspecies transmission,

including transmission to humans. Additional studies are needed to characterize the other viral and cellular genes involved in transmission.

Because influenza viruses possessing H5N1 gene segments have the capacity to be transmitted to mammals, they have the potential to cause pandemics. We therefore determined the efficacy of the newly approved NA inhibitor zanamivir against these viruses in vitro and in vivo. Because previous studies have established that zanamivir is efficacious against the A/HK/156/97 (H5N1) virus in vitro and in the mouse, we used A/HK/156/97 (H5N1) as a reference strain in establishing the efficacies of zanamivir against the H6N1 and H9N2 influenza viruses. Our experiments established that the viral replication and NA activities of A/Quail/HK/G1/97 (H9N2), A/Teal/HK/W312/97 (H6N1), and A/Chicken/HK/G9/97 (H9N2) are all inhibited in vitro by zanamivir. The results of the ELISAs indicated that the EC₅₀ were 8.5 to 14 μ M, and the results of the NA inhibition assays indicated that the IC₅₀ were 5 to 10 nM. The susceptibilities of these avian isolates are within the ranges reported previously from NA assays and plaque reduc-

tion assays for human strains and clinical isolates (39). In this study, ELISA-based yield reduction assays were used rather than plaque assays to determine IC_{50} . In yield reduction assays the IC_{50} determined are virus input dependent and tend to be higher than values determined by plaque reduction (34). Therefore, these data indicate that all five avian isolates are highly susceptible in vitro to zanamivir.

Comparison of the in vivo efficacies of zanamivir in this study with those of previous studies with human influenza strains is difficult because in vivo efficacy is dependent on virus input, which would be different between studies. Overall efficacy appears lower for these avian strains, but this could relate to the relatively high pathogenicities of avian strains in the mouse model compared with those of human strains (30). For comparisons of efficacies between the different avian isolates, dosing was initiated before infection, since this ensures greater reproducibility. Differences in viral sensitivity to zanamivir were detected in the mouse model; A/Quail/HK/G1/97 (H9N2) and A/Chicken/HK/G1/97 (H9N2) were more sensitive to zanamivir than A/Teal/HK/W312/97 (H6N1) and A/HK/156/97 (H5N1). This difference may be due to the interplay between the HAs and NAs of these viruses or to the affinity of binding of the HA (21). In addition, both the A/HK/156/97 (H5N1) and A/Teal/HK/W312/97 (H6N1) viruses have a 19-amino-acid deletion in the NA stalk that results in a decreased ability of viruses to escape from cells (18). Possibly, this deletion in vivo may have some unknown advantage in the presence of NA inhibitors. Zanamivir completely abolished the transmission of influenza virus to the brains of mice even at the lowest doses tested (1 mg/kg); this finding indicates that systemic spread may be related to the level of viral replication in the lungs. At high levels of replication, there is possibly sufficient pulmonary damage for virus to reach the circulation and, if levels are high enough, to reach the brain. The mechanism(s) for spread through the blood-brain barrier is not clearly understood and is probably related to a specific viral protein(s). Although neurologic disorders associated with influenza in humans are rare, there are reports of neurologic disorders associated with H3N2 and B strains of human influenza virus (23, 24, 32, 35). Although the Spanish influenza virus of 1918 has not been causally associated with encephalitis lethargica, the question remains open until further evidence is accumulated.

Although zanamivir significantly reduced the levels of A/Quail/HK/G1/97 (H9N2) and A/Teal/HK/W312/97 (H6N1) in the lungs of mice, it did not completely inhibit virus replication. Similar findings were reported previously when zanamivir was tested against A/HK/156/97 (H5N1) and other influenza viruses in the mouse system (11, 13, 28). These results may relate to the high levels of virus replication in this model overwhelming the inhibitor in experiments in which zanamivir produced up to a 4-log₁₀ EID₅₀ reduction in virus growth. One possible explanation is that zanamivir-resistant mutants were appearing, but the present and previous studies indicate that the viruses sampled late in infection were as sensitive to zanamivir as the original parental virus. This result may also suggest that the virus can evade contact with the drug by some mechanism, possibly by cell-to-cell spread.

A new pandemic of influenza is considered to be inevitable (26, 38). Because of this inevitability and because of the efficacy of zanamivir against H5N1, H6N1, and H9N2 avian vi-

rus, it is now important to determine the long-term stability of both zanamivir and oseltamivir so that consideration can be given to stockpiling these agents to prepare for future pandemics.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants AI08831 and AI95357 from the National Institute of Allergy and Infectious Diseases, by Cancer Center Support (CORE) grant CA-21765, and by the American Lebanese Syrian Associated Charities (ALSAC).

Mikhail N. Matrosovich and Yi Guan provided valuable consultation; the viruses were provided by Kennedy Shortridge from Hong Kong University. We thank Glaxo Wellcome Research and Development for providing the NA inhibitor zanamivir, Scott Krauss and Melissa Norwood for technical assistance, Julia Cay Jones for editing the manuscript, and Alice Herren for typing the manuscript.

REFERENCES

1. Aymard-Henry, M., M. T. Coleman, W. R. Dowdle, W. G. Laver, G. C. Schild, and R. G. Webster. 1973. Influenza virus neuraminidase-inhibition test procedures. *Bull. W. H. O.* **48**:199-202.
2. Belshe, R. B., M. H. Smith, C. B. Hall, R. Betts, and A. J. Hay. 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. *J. Virol.* **62**:1508-1512.
3. Centers for Disease Control and Prevention. 1998. Update: isolation of avian influenza A (H5N1) viruses from humans—Hong Kong, 1997-1998. *Morb. Mortal. Wkly. Rep.* **46**:1245-1247.
4. Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* **351**:472-477.
5. de Jong, J. C., E. C. Claas, A. D. Osterhaus, R. G. Webster, and W. L. Lim. 1997. A pandemic warning? *Nature* **389**:554.
6. Dybing, J. K., S. Schulz-Cherry, D. E. Swayne, D. L. Suarez, and M. L. Perdue. 2000. Distinct pathogenesis of Hong Kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. *J. Virol.* **74**:1443-1450.
7. Gao, P., S. Watanabe, T. Ito, H. Goto, K. Wells, M. McGregor, A. J. Cooley, and Y. Kawaoka. 1999. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. *J. Virol.* **73**:3184-3189.
8. Grunert, R. R., J. W. McGahen, and W. L. Davies. 1965. The *in vivo* antiviral activity of 1-adamantadine (amantadine). *Virology* **26**:262-269.
9. Guan, Y., K. F. Shortridge, S. Krauss, and R. G. Webster. 1999. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? *Proc. Natl. Acad. Sci. USA* **96**:9363-9367.
10. Guan, Y., K. F. Shortridge, S. Krauss, P. S. Chin, K. C. Dyrting, T. M. Ellis, R. G. Webster, and M. Peiris. 2000. H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. *J. Virol.* **74**:9372-9380.
11. Gubareva, L. V., M. N. Matrosovich, M. K. Brenner, R. C. Bethell, and R. G. Webster. 1999. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. *J. Infect. Dis.* **178**:1257-1262.
12. Gubareva, L. V., C. R. Penn, and R. G. Webster. 1995. Inhibition of replication of avian influenza viruses by the neuraminidase inhibitor 4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid. *Virology* **212**:323-330.
13. Gubareva, L. V., R. Bethell, G. J. Hart, K. G. Murti, C. R. Penn, and R. G. Webster. 1996. Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. *J. Virol.* **70**:1818-1827.
14. Gubareva, L. V., J. A. McCullers, R. C. Bethell, and R. G. Webster. 1998. Characterization of influenza A/Hong Kong/156/97 (H5N1) virus in a mouse model and protective effect of zanamivir on H5N1 infection in mice. *J. Infect. Dis.* **178**:1592-1596.
15. Guo, Y. J., J. W. Li, and I. Cheng. 1999. Discovery of humans infected by avian influenza A (H9N2) virus. *Chin. J. Exp. Clin. Virol.* **15**:105-108.
16. Hayden, F. G., J. J. Treanor, R. F. Betts, M. Lobo, J. D. Esinhart, and E. K. Hussey. 1996. Safety and efficacy of the neuraminidase inhibitor GG167 in experimental human influenza. *JAMA* **275**:295-299.
17. Hayden, F. G., B. S. Rollins, and A. J. Hay. 1990. Anti-influenza virus activity of the compound LY253963. *Antivir. Res.* **14**:25-38.
18. Hoffmann, E., J. Stech, I. Leneva, S. Krauss, C. Scholtissek, P. S. Chin, M. Peiris, K. F. Shortridge, and R. G. Webster. 2000. Characterization of the influenza A gene pool in avian species in southern China: was H6N1 a

- derivative or a precursor of H5N1? *J. Virol.* **74**:6309–6315.
19. Lin, Y. P., M. Shaw, V. Gregory, K. Cameron, W. Lim, A. Klimov, K. Subbarao, Y. Guan, S. Krauss, K. Shortridge, R. Webster, N. Cox, and A. Hay. 2000. Avian-to-human transmission of H9N2 subtype influenza A viruses—relationship between H9N2 and H5N1 human isolates. *Proc. Natl. Acad. Sci. USA* **97**:9654–9658.
 20. Lu, X., T. M. Tumpey, T. Morken, S. Zaki, N. Cox, and J. Katz. 1999. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. *J. Virol.* **73**:5903–5911.
 21. Matrosovich, M., N. Zhou, Y. Kawaoka, and R. G. Webster. 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. *J. Virol.* **73**:1146–1155.
 22. McCauley, J. W., L. A. Pullen, M. Forsyth, C. R. Penn, and G. P. Thomas. 1995. 4-Guanidino-Neu5Ac2en fails to protect chickens from infection with pathogenic avian influenza virus. *Antivir. Res.* **27**:179–186.
 23. McCullers, J., S. Facchini, P. J. Chesney, and R. G. Webster. 1999. Influenza B virus encephalitis. *Clin. Infect. Dis.* **28**:898–900.
 24. Paisley, J. W., F. W. Bruhn, B. A. Lauer, and K. McIntosh. 1978. Type A2 influenza viral infections in children. *Am. J. Dis. Child.* **132**:34–36.
 25. Patriarca, P. A., and N. J. Cox. 1997. Influenza pandemic preparedness plan for the United States. *J. Infect. Dis.* **176**(Suppl. 1):S4–S7.
 26. Peiris, M., K. Y. Yuen, C. W. Leung, K. H. Chan, P. L. S. Ip, R. W. M. Lai, W. K. Orr, and K. F. Shortridge. 1999. Human infection with influenza H9N2. *Lancet* **354**:916–917.
 27. Rott, R., M. Orlich, and C. Scholtissek. 1979. Correlation of pathogenicity and gene constellation of influenza A viruses. III. Non-pathogenic recombinants derived from highly pathogenic parent strains. *J. Gen. Virol.* **44**:471–477.
 28. Ryan, D. M., J. Ticehurst, M. H. Dempsey, and C. R. Penn. 1994. Inhibition of influenza virus replication in mice by GG167 (4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid) is consistent with extracellular activity of viral neuraminidase (sialidase). *Antimicrob. Agents Chemother.* **38**:2270–2275.
 29. Scholtissek, C., I. Koennecke, and R. Rott. 1978. Host range recombinants of fowl plague (influenza A) virus. *Virology* **91**:79–85.
 30. Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster. 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. *Virology* **252**:331–342.
 31. Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox. 1998. Characterization of an avian influenza A (H5N1) virus isolated from child with fatal respiratory illness. *Science* **279**:393–396.
 32. Sweet, C., and H. Smith. 1980. Pathogenicity of influenza virus. *Microbiol. Rev.* **44**:303–330.
 33. Thomas, G. P., M. Forsyth, C. R. Penn, and J. W. McCauley. 1994. Inhibition of the growth of influenza viruses *in vitro* by 4-guanidino-2,4-dideoxy-*N*-acetylneuraminic acid. *Antivir. Res.* **24**:351–356.
 34. Tisdale, M. 2000. Monitoring of viral susceptibility: new challenges with the development of influenza NA inhibitors. *Rev. Med. Virol.* **10**:45–55.
 35. Togashi, T. Y., M. Matsuzono, M. Anakura, and K. Nerome. 1997. Acute encephalitis and encephalopathy at the height of influenza in childhood. *Nippon Rinsho* **55**:2699–2705.
 36. von Itzstein, M., W.-Y. Wu, G. B. Kok, M. S. Pegg, J. C. Dyason, B. Jin, T. Van Phan, M. L. Smythe, H. F. White, S. W. Oliver, P. M. Colman, J. N. Varghese, D. M. Ryan, J. M. Woods, R. C. Bethell, V. J. Hotham, J. M. Cameron, and C. R. Penn. 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **363**:418–423.
 37. Ward, A. C. 1997. Virulence of influenza A virus for mouse lung. *Virus Genes* **14**:187–194.
 38. Webster, R. G. 1997. Predictions for future human influenza pandemics. *J. Infect. Dis.* **176**(Suppl. 1):S14–S19.
 39. Woods, J. M., R. C. Bethell, J. A. Coates, N. Healy, S. A. Hiscox, B. A. Pearson, D. M. Ryan, J. Ticehurst, J. Tilling, S. M. Walcott, and C. R. Penn. 1993. 4-Guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid is a highly effective inhibitor both of the sialidase (neuraminidase) and of growth of a wide range of influenza A and B viruses *in vitro*. *Antimicrob. Agents Chemother.* **37**:1473–1479.
 40. Zhou, N. N., K. F. Shortridge, E. C. Claas, S. L. Krauss, and R. G. Webster. 1999. Rapid evolution of H5N1 influenza viruses in chickens in Hong Kong. *J. Virol.* **73**:3366–3374.