The pH of Activation of the Hemagglutinin Protein Regulates H5N1 Influenza Virus Pathogenicity and Transmissibility in Ducks

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Running Title: H5N1 HA protein in pathogenesis and transmissibility

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Abstract word count: 250

Text word count: 5369

Keywords: influenza / ducks / virulence / membrane fusion / pH
ABSTRACT

While the molecular mechanism of membrane fusion by the influenza virus hemagglutinin (HA) protein has been studied extensively in vitro, the role of acid-dependent HA protein activation in virus replication, pathogenesis, and transmission in vivo has not been characterized. To investigate the biological significance of the pH of activation of the HA protein, we compared the properties of four recombinant viruses with altered HA protein acid stability to those of wild-type A/chicken/Vietnam/C58/04 (H5N1) in vitro and in mallards. Membrane fusion by wild-type was activated at pH 5.9. Wild-type virus had a calculated environmental persistence of 62 days and caused extensive morbidity, mortality, shedding, and transmission in mallards. An N114K mutation that increased the pH of HA activation by 0.5 units resulted in decreased replication, genetic stability, and environmental stability. Changes of +0.4 and –0.5 units in the pH of activation by Y23H and K58I mutations, respectively, reduced weight loss, mortality, shedding, and transmission in mallards. An H24Q mutation that decreased the pH of activation by 0.3 units resulted in weight loss, mortality, clinical symptoms, and shedding similar to wild-type. However, the HA-H24Q virus was shed more extensively into drinking water and persisted longer in the environment. The pH of activation of the H5 HA protein plays a key role in the propagation of H5N1 influenza viruses in ducks and may be a novel molecular factor in the ecology of influenza viruses. The data also demonstrates that H5N1 neuraminidase activity increases the pH of activation of the HA protein in vitro.
INTRODUCTION

Highly pathogenic H5N1 influenza viruses were transmitted to humans in 1997 in Southeast Asia (7) and have subsequently spread across Asia, Europe, and Africa (53). Millions of poultry have been culled to control outbreaks (19), and more than 250 human lives have been lost (http://www.who.int/csr/disease/avian_influenza/en/). These viruses appear currently to lack the molecular properties required for sustained transmission among humans. There is an urgent need to understand the molecular properties that contribute to the transmission and host range of these viruses for their effective surveillance and containment.

The transmissibility and pathogenicity of influenza A viruses, including the H5N1 subtype, in avian and mammalian species are determined by both viral and host factors (8, 39). One key factor is the multifunctional hemagglutinin (HA) protein. During viral entry, the HA protein binds to sialic acid–containing receptors on host cells; the virus then undergoes endocytosis and its HA protein is activated at low pH to cause the fusion of the viral and endosomal membranes (11, 41). The host range of influenza A viruses depends in a large part on the receptor specificity of the HA protein. Avian influenza viruses generally bind to α(2,3) sialosides with greater affinity, while human influenza viruses usually bind to α(2,6) sialosides with greater affinity (4, 37). The receptor binding affinities and specificities of HA proteins also depend on internal linkages and modifications of inner oligosaccharides, and glycan microarray profiling has revealed differences in receptor binding between seasonal human influenza viruses and H5N1 viruses (23, 45, 46). Thus, the natural distribution of various sialosides in different tissues of different species helps to determine both tissue tropism and species specificity (31, 40, 50, 58). The post-translational cleavability of the HA0 precursor protein into the fusion-capable HA1-HA2 complex is a critical determinant of the virulence of influenza viruses (16, 22, 55). The presence of a polybasic cleavage site in H5 and H7 influenza viruses allows HA protein
cleavage in the trans-Golgi network by ubiquitous furin-like enzymes and is a marker of high pathogenicity (12, 38, 55).

During entry into host cells, influenza viruses are exposed to increasingly lower pH until a threshold is reached at which HA protein trimers undergo irreversible conformational changes that promote membrane fusion (11, 41). Threshold pH values differ among influenza viruses, and a change in the pH of fusion of the HA protein can help influenza viruses to adapt to different cell lines (5, 25) and host species (13) or to the higher endosomal pH induced by high concentrations of the antiviral drug amantadine (6, 9, 42-44). In general, a high pH of HA protein activation could result in influenza virus inactivation in the environment or during transport to the cell surface for intracellularly cleaved HA proteins (2, 44). On the other hand, a low pH of HA protein activation could result in degradation in the lysosome as the pH of the endocytic pathway decreases from early endosomes to late endosomes to lysosomes (61). Therefore, for efficient propagation within a biological host and ecological niche, an influenza virus may have an optimal range of pH of activation for the HA protein. Moreover, the optimal activation pH may change upon introduction of an influenza virus into a new host species or environment.

Aquatic birds are a natural reservoir of influenza viruses, but surprisingly little is known about the molecular basis of influenza virus propagation in these species. To test the hypothesis that the pH of activation of the HA protein contributes to the pathogenicity and transmissibility of H5N1 influenza viruses in the mallard, a prototypic aquatic bird, we previously generated four recombinant H5N1 viruses containing mutations that altered the acid stability of the HA protein without changing its level of expression, cleavage, receptor binding, or membrane fusion efficiency (36). Two of the mutations increased the pH of membrane fusion of the H5N1 HA protein (Y231H and N1142K) and the other two mutations reduced the pH of fusion (H241Q and K582I). HA1 mutations Y231H and H241Q (H5 numbering) are located in the fusion peptide...
pocket and were originally chosen because of their presence in H1 and H9 subtypes, respectively. The K58I mutation in the A-helix of HA2 was chosen because it decreases the pH of membrane fusion of the H3 HA protein by 0.7 units (44). The N114K mutation in the fusion peptide pocket was chosen because it increases the pH of membrane fusion by approximately 0.5 units in H3 and H7 subtypes (6). Here we measured the effects of the H5 HA protein mutations on virus replication in vitro, on genetic stability after repeated passage in eggs, and on environmental stability. Mallards were inoculated with the recombinant viruses and housed with contact ducks to determine the effects of the mutations on virus shedding, pathogenesis, and transmissibility. An H24Q mutation in the HA protein was found to decrease the pH of activation by 0.3 pH units, increase the titers of infectious virus recovered from ducks’ water dishes, and prolong the persistence of infectious virus in the environment. In general, changes in the acid stability of the HA protein were found to alter H5N1 influenza virus replication, pathogenicity, and transmissibility.
MATERIALS AND METHODS

Viruses, plasmids and cell culture. Recombinant viruses and plasmids containing HA protein mutations Y231H, H241Q, K583I and N1142K were generated previously (36). All experiments using H5N1 influenza viruses were performed in a USDA-approved biosafety level 3+ containment facility. Monolayer cultures of Vero cells (ATCC CCL-81) were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin. Monolayers of Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were grown in Minimum Essential media (MEM) supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin.

Virus growth kinetics. Single-step growth curves in MDCK cells were determined for each of the recombinant viruses. Confluent monolayers were infected at a multiplicity of infection (MOI) of ~3 PFU per cell. After 1 hour incubation, cells were washed with 0.9% aqueous NaCl solution (pH 2.2) to remove any free infectious virus particles and then washed twice with phosphate-buffered saline (PBS) to adjust the pH. Cells were incubated at 37°C in MEM (containing 4% bovine serum albumin and 1% glutamine). Supernatants were collected 2, 4, 6, 8, and 10 hours postinfection and stored at −70 °C. To determine multiple-step growth kinetics, MDCK cells were infected at an MOI of ~0.01 PFU/cell. After 1 hour incubation, cells were washed twice with PBS and incubated at 37°C in MEM (containing 4% bovine serum albumin and 1% glutamine). Supernatants were collected 12, 24, 36, 48, 60, and 72 hours postinfection and stored at −70 °C. Virus was titrated as described previously (60). Briefly, confluent MDCK cells were incubated for 1 hour at 37°C with 10-fold serial dilutions of virus in 1 ml infection medium. The cells were then washed and overlaid with freshly prepared MEM containing 0.3% BSA and 0.9%
Bacto agar. After 37°C incubation for 3 days, plaques were visualized by using 0.1% crystal violet solution containing 10% formaldehyde.

Genetic stability. The H5N1 influenza viruses were serially passaged in 10-day old embryonated chicken eggs to assess the genetic stability of the introduced mutations. Eggs were infected with 1 HA unit of sequence-confirmed virus. Allantoic fluid was collected and the HA titer was measured to determine dilution for subsequent passage of the virus. RNA was extracted and sequenced as described above.

Environmental stability. Stocks of recombinant viruses were diluted 1:50 in distilled water (pH 7.4) containing 2 mM HEPES buffer. Aliquots were incubated at 28 °C (the approximate environmental temperature in Louisiana during summer, allowing comparison with data from similar studies) (2). Aliquots were removed daily for 8 days and compared to initial virus titer (PFU/ml) by plaque assay. The sequential data was log₁₀ transformed and analyzed by linear regression using Graphpad Prism software (Graphpad Software, La Jolla, CA). The gradient from this model was then used to calculate the estimated persistence of 1x10⁶ PFU/ml of recombinant virus, and the time required to reduce the infectivity of the initial inoculum by 90% (1log₁₀). Differences in the linear regression models were measured by using the Graphpad Prism software.

Inoculation and transmission studies in mallards. Groups of three 4-week-old mallards (Anas platyrhynchos) were inoculated via intranasal, intraocular, and intratracheal instillation of ~10⁶ EID₅₀ of virus in a 1-ml volume, as described previously (21). Two uninoculated contact ducks were placed in the cage with the inoculated ducks 24 hours post-inoculation (p.i.), and shared a cage with the inoculated ducks 24 hours post-inoculation.
common food and water source. Birds were weighed and observed daily for signs of morbidity or mortality over a period of 14 days. Birds that did not eat or drink on their own due to severe disease signs were euthanized, and their deaths were recorded on the following day of observation. Tracheal and cloacal swabs were collected from all ducks on days 3, 5, 7 and 10 p.i., and 0.5 ml of drinking water was sampled on days 1, 3, 5, 7 and 10 days p.i.. Influenza virus was detected by virus isolation in 10-day-old embryonated chicken eggs as previously described (14, 47). Virus was titrated in positive samples by calculating the EID\(_{50}\), using the method of Reed and Muench (35); the lower limit of quantification was 0.75 log\(_{10}\) EID\(_{50}/ml.\) Swab samples with detectable influenza virus but titers below the limit of quantification were reported as having a titer of <10\(^{1}\) EID\(_{50}/ml.\) All data shown were derived from two separate experiments. All animal experiments were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital and performed in compliance with relevant institutional policies, the Association for the Accreditation of Laboratory Animal Care guidelines, the National Institutes of Health regulations, and local, state, and federal laws.

**Transient expression of HA and NA proteins.** Monolayers of Vero cells in 6-well dishes (85–95% confluence) were transiently transfected with 1 µg of pCAGGS A/chicken/Vietnam/C58/04 HA DNA, using the Lipofectamine Plus expression system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transfected Vero cells were incubated for 4 hour at 37°C. DMEM (containing 10% fetal bovine serum and 1% glutamine) was then added to cells, and cells were incubated for 16 hours at 37°C. Cells were then treated as indicated for each experiment. NA protein was expressed by using 0.1-1.0 µg pCAGGS A/chicken/Vietnam/C58/04 NA plasmid.
**Syncytium assay.** Monolayers of Vero cells grown in 6-well plates were transfected with 1.0 µg pCAGGS HA as described above, or infected with recombinant virus at a multiplicity of infection (MOI) of ~3 plaque forming units (PFU) per cell. At 16 hours post-transfection or 6 hours post-infection, cell monolayers were overlaid for 5 min with phosphate-buffered saline with magnesium and calcium (PBS+) that was adjusted to the reported pH with a 0.1 pH unit resolution using 0.1 M citric acid. Cells were neutralized by using DMEM (containing 10% fetal bovine serum and 1% glutamine) and incubated at 37 °C for 2 h. Samples were fixed and stained with a Hema 3 stat pack staining kit (Fisher) according to the manufacturer's instructions. Representative microscopic fields were captured with a Nikon D70 digital camera attached to a Nikon Eclipse TS100 inverted microscope (26).

**NA activity assay and NA inhibition.** A modified fluorometric assay was used to determine the enzymatic activity of the NA protein present in transfected cell lysates with the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma) (15, 34, 59). The fluorescence of the released 4-methylumbelliferone was measured in a Fluoroskan II spectrophotometer (Labsystems, Helsinki, Finland) using excitation and emission wavelengths of 355 and 460 nm, respectively. NA protein enzymatic activity was standardized to 0.1 mg total protein by using a BCA assay (Sigma, St. Louis, MO) and was expressed as the quantity of substrate (picomoles) converted during a 30-min incubation at 37 °C. The NA inhibitor oseltamivir carboxylate ([3R,4R,5S]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexene-1-carboxylic acid) was provided by Hoffmann-La Roche, Ltd. (Basel, Switzerland). The compound was dissolved in distilled water, and aliquots were stored at −20°C until used. NA activity was inhibited by using a 4 µM concentration of oseltamivir carboxylate added immediately post-
transfection or 1 hour before the assay. The effect of NA protein inhibitor on the pH of fusion was determined by performing a syncytia-formation assay in parallel.
RESULTS

The HA protein mutations have little effect on the in vitro replication kinetics of recombinant H5N1 influenza viruses. In a previous study, we identified four mutant H5 HA proteins whose pH values of membrane fusion differed from that of wild-type HA protein when expressed from transiently transfected plasmid DNA (36). Here we determined whether the HA protein mutations affected the in vitro replication kinetics of recombinant H5N1 influenza viruses by generating single-step and multiple-step growth curves. Single-step growth curves showed that mutant and wild-type viruses grew at similar rates over the 10-hour time course (Figure 1A). In multiple-step growth curves, viruses containing the HA protein mutations Y231H, H241Q and K582I had replication rates similar to wild-type virus (Figure 1B). Titers of the virus containing an N1142K mutation in the HA protein were similar to those of wild-type virus at the 12-hour time point but were later reduced by 1-3 log10.

The HA protein mutations alter the pH of membrane fusion in vitro. The cell-surface expression, cleavage, receptor binding affinities, and membrane fusion efficiencies of the mutant HA proteins in Vero cells were previously found to be similar to those of wild-type virus (36). Moreover, infection of the viruses in DF-1 primary chicken embryonic fibroblasts resulted in similar HA protein properties as when the viruses were infected in Vero cells. To determine the effects of the mutations on the pH of membrane fusion, monolayers of MDCK cells were infected with recombinant H5N1 influenza viruses at an MOI of ~3 PFU/cell and then exposed to PBS solutions of varying pH, at a resolution of 0.1 units. The highest pH at which cell-cell membrane fusion was induced in cells infected with wild-type virus was 5.9 (Figure 2). The mutations H241Q and K582I reduced the pH of membrane fusion to 5.6 and 5.4, respectively,
while the Y231H and N1142K mutations increased the pH of membrane fusion to 6.3 and 6.4, respectively. The N1142K mutation which increased the pH of HA activation by 0.5 pH units resulted in decreased virus fitness over several cycles of replication in vitro, while the other mutations did not alter in vitro replication kinetics.

The N1142K mutation is genetically unstable over multiple passages in eggs. To test the genetic stability of the HA protein mutations, wild-type and mutant viruses were passaged 10 times in 10-day old embryonated chicken eggs. The sequence identity of each of the passage 1 (P1) recombinant viruses had been confirmed previously (36). Purified viral RNA sampled from allantoic fluid at P5 and P10 was sequenced. In parallel, syncytia formation assays were performed using Vero cells infected with P10 viruses to determine whether repeated passage in eggs resulted in any mutations that might alter the acid stability of the viral HA proteins (Table 1). Wild-type virus and viruses containing HA protein mutations H241Q and K581I showed no additional mutations over the course of 10 passages and no change in the pH of membrane fusion. Virus containing the Y231H mutation maintained the mutation for at least 5 passages in eggs and acquired an additional HA protein mutation, R2281I, between P5 and P10. Residue R228 (H5 numbering) is located in the receptor-binding pocket of the HA1 subunit with its sidechain facing away from the pocket (46, 57) such that the R2281I mutation may enhance receptor binding in eggs (56). Despite the extra R2281I mutation, the P10 virus caused membrane fusion at a pH of 6.3, as did P1 Y231H virus without the additional R2281I mutation. The virus containing the N1142K mutation in the HA protein was the only recombinant virus that had an altered pH of membrane fusion at P10 compared to the P1 stock virus, a decrease from pH 6.4 to 6.1 (Table 1). Both P5 and P10 K1142N viruses showed reversion mutations, demonstrating that the N1142K mutation was not genetically stable and was selected against within 5 passages.
Changes in the pH of activation of the HA protein can alter the environmental stability of H5N1 influenza viruses. The environmental stability of highly pathogenic H5N1 isolates has been found to be lower than that of lower-pathogenicity viruses (1, 2). To determine whether changes in the pH of fusion of the HA protein alter the environmental stability of H5N1 viruses, we incubated the viruses in the present study at 28 °C for 8 days and measured virus titer as a function of time by plaque assay. Data from each series was plotted, and the gradient of virus degradation was calculated by linear regression analysis (Table 2). Wild-type virus and the virus containing a Y231H mutation in the HA protein showed a similar rate of titer reduction (1 log$_{10}$ every 10 days), a rate of degradation that matches those of other highly pathogenic H5N1 isolates (2). This result suggests that changes in the pH of fusion as great as +0.4 pH units can be tolerated without a loss in environmental stability. Viruses containing H241Q and K582I mutations, both of which promoted membrane fusion at lower pH values than wild-type virus, were calculated to lose 1 log$_{10}$ in titer every 13 days. Thus, the two mutant viruses with lower pH values of activation retained infectivity longer than the wild-type virus. The virus containing an N1142K mutation rapidly lost infectivity in the environmental stability experiment, losing 1 log$_{10}$ in titer approximately every 2 days (Table 2). Therefore, an increase in the pH of HA activation to 6.4 due to the N1142K mutation resulted in greatly reduced environmental stability, and a decrease in the pH of activation of the HA protein to 5.6 or 5.4 due to the H241Q and K582I mutations, respectively, moderately increased environmental stability.

The pH of activation of the HA protein contributes to the pathogenicity and transmissibility of H5N1 viruses in mallards. To measure the biological properties of the
mutant viruses in mallards, we inoculated duplicate groups of three animals and introduced two contact animals into the cage of each group after one day. Wild-type and H24;Q viruses induced considerable weight loss in both inoculated and contact animals (Figure 3) and caused death in 60% and 70%, respectively, of animals (Table 3). Conversely, the Y23;H and K58;I viruses did not induce weight loss or death in either inoculated or contact animals. Moreover, the Y23;H virus caused only cloudy eyes in 50% of the inoculated ducks, while the K58;I virus caused cloudy eyes in only one contact duck. While the virus containing an N114;K mutation in the HA protein did not induce weight loss or death in inoculated ducks, contact animals in this group unexpectedly showed weight loss after 4 days, and three of the four contact animals died. Neurological signs were observed in these contact animals, whereas none were observed in the inoculated group. Because of these unexpected findings, we sequenced viral RNA isolated from positive swabs from surviving inoculated and contact birds on days 7 and 10. In all cases, the N114;K mutation had reverted to the wild-type N114;2, as it had after serial passage in eggs (Table 1). This reversion offers the most plausible explanation for the increased transmissibility and pathogenicity in contact birds in the N114;2 group. The reversion mutation also explains the greater weight loss, morbidity, and mortality in the contact birds than in the infected birds in this group. No other reversion mutations were sequenced from swabs of contact ducks infected with the other viruses including H24;Q.

To assess replication and transmission potential, titers of virus shed from the trachea and cloaca were measured (Table 4). Inoculated birds in all groups shed virus on day 3; therefore, all of the viruses were capable of initial infection and replication. However, even at this early time point, the viruses containing HA protein mutations Y23;H and K58;I productively infected fewer ducks than wild-type and H24;Q viruses. Wild-type and H24;Q viruses were shed at similar levels on days 3 and 5. On day 7, wild-type virus was not shed, whereas H24;Q virus continued
to be shed. All contact birds in the wild-type and HA24,Q groups were shedding virus by day 3 p.i. (100% transmission). All contact birds in the H24,Q group succumbed to infection, whereas only half of the contact birds in the wild-type group died. The Y23,H virus was not detected in any contact birds throughout the experiment, showing that the mutation results in attenuated transmission as compared to that of wild-type virus. Five days p.i., the K58,I virus was detected in one inoculated bird and one contact bird, showing that its fitness and transmissibility were lower than that of wild-type virus. The N114,K virus showed inconsistent shedding in inoculated birds. Inoculated birds shed virus on day 3 but not on day 5, yet some birds again shed virus on days 7 and 10. This result suggests that transmission to contact birds was mediated by the reverted K114,N virus, which was then transmitted back to inoculated ducks before being detected on days 7 and 10. On day 3 p.i., tracheal shedding was generally observed more often and at higher titers than cloacal shedding, consistent with previous work (48). The H24,Q and K58,I mutations did not appear to increase cloacal virus shedding, and therefore small decreases in the pH of activation (and inactivation) of the HA protein may be insufficient to enhance virus replication in the low-pH environment of the duck digestive tract (49).

We also investigated shedding of the recombinant viruses by titrating virus in the ducks’ water dishes. Wild-type virus was detected on days 1, 3 and 5 p.i. and had a peak titer of 3.25 log$_{10}$ EID$_{50}$ on day 3 (Table 5). No Y23,H virus was detected on any day, consistent with low shedding of this virus on day 3 and none on days 5, 7, and 10 p.i.. The K58,I virus titer in the water dishes was comparable to that of wild-type virus on days 1 and 3 but was subsequently undetectable, consistent with the pattern of virus shedding from the trachea and cloaca of ducks (Table 4). The presence of N114,K virus in water dishes on days 3 and 5 but not on day 1 is consistent with low-level shedding until after reversion. Higher titers of the H24,Q virus than that of wild-type virus were detected in the water dishes on days 1 through 7, consistent with this
mutant’s greater environmental stability and lethality in contact ducks. Overall, our results show that reduction of the pH of membrane fusion in the virus containing an H241Q HA mutation enhances two properties that could promote H5N1 virus transmission in aquatic birds: shedding of virus into water and persistence of virus infectivity in water.

NA activity promotes pH-mediated membrane fusion induced by the HA protein. The highest pH at which wild-type virus caused membrane fusion was 5.9 (Figure 2); however, we previously found that wild-type HA protein expressed from transiently transfected plasmid DNA caused membrane fusion only when the pH was decreased to 5.5 (36). The occurrence of this change in the context of virus infection (with expression of all viral proteins) in the present study suggested that one or more of the other viral proteins promotes acid-induced activation of the H5N1 HA protein. Previous studies have shown that the NA protein facilitates entry of H3N2 influenza viruses (28, 32). To determine whether NA protein expression increases the pH of membrane fusion by the HA protein, we transfected Vero cells with the pCAGGS HA wild-type plasmid in the presence and absence of co-transfection with the pCAGGS NA wild-type plasmid. Titration showed that 0.1 µg of plasmid DNA produced neuraminidase activity similar to that in 10 µl of allantoic fluid containing virus (data not shown); therefore, a 1:0.1 µg ratio of HA:NA was used in all follow-up experiments. Transfected cells expressing the wild-type H5N1 HA and NA surface proteins showed syncytia formation at pH 5.9, the same pH as wild-type virus (Figure 4A). Having established that expression of the NA protein accounted for the observed increase in the pH of HA-mediated membrane fusion, we next examined whether NA enzymatic activity was responsible for the increase. NA enzymatic activity was eliminated in Vero cells co-transfected with pCAGGS HA and NA plasmids by treatment with oseltamivir carboxylate (3, 51) (Figure 4B). The syncytium formation assay was repeated using cells co-expressing the HA
and NA proteins in the presence of oseltamivir carboxylate. When NA enzymatic activity was inhibited by the drug, the pH of HA-mediated membrane fusion decreased to pH 5.5, the same value observed in cells expressing HA protein alone (Figure 4A). These results are consistent with the promotion of H5 HA-mediated membrane fusion by N1 neuraminidase activity.
DISCUSSION

To investigate how the pH of activation of the HA protein influences the in vitro and in vivo properties of influenza viruses, we compared four recombinant viruses with altered pH-dependent HA protein stability to wild-type A/chicken/Vietnam/C58/04 (H5N1) virus. An N1142K mutation in the HA2 fusion peptide pocket region increased the activation pH of the HA protein from 5.9 to 6.4, allowing activation at mildly acidic conditions. This mutation dramatically reduced the fitness of the virus in three ways: (1) multiple-step replication in vitro was reduced by a factor greater than 10; (2) infectivity in the environment decreased 4 times as rapidly as that of wild-type virus; and (3) the virus reverted to the wild-type sequence within 5 passages in chicken eggs and after inoculation in mallards. The N1142K mutation may increase the pH of activation of the HA protein above the threshold pH at which a significant portion of intracellularly cleaved HA trimers become prematurely triggered, and inactivated, during transport to the cell surface (44). The HA protein mutations Y23H and K58I changed the activation pH of the HA protein to 6.3 and 5.4, respectively. While these two mutations had opposite effects on the activation pH, the recombinant viruses bearing the mutations had similar phenotypes. Despite in vitro replication rates similar to that of wild-type virus, the viruses bearing Y23H and K58I mutations did not induce weight loss, neurological signs, or mortality in mallards, were not efficiently transmitted, and were shed significantly less. Overall, the data show that efficient and sustainable infection of mallards by H5N1 influenza virus is not supported by HA protein activation pH values less than 5.5 or greater than 6.2.

The results of experiments with the virus bearing an HA-H24Q mutation suggest that robust infection in mallards is supported by activation pH values between 5.6 and 5.9. The data also raise the possibility that natural mutations that slightly reduce the pH of activation of the HA
proteins could increase the transmission of H5N1 influenza viruses among mallards. The wild-type virus and the HA-H241Q virus had similar replication kinetics in vitro and induced similar weight loss, mortality, clinical signs, and shedding in mallards, but higher titers of H241Q virus were found in the ducks’ water dishes, and the H241Q virus retained infectivity ~20% longer than wild-type virus in an environmental stability experiment. The fact that all of the contact ducks succumbed to infection with transmitted H241Q virus while only half died from transmitted wild-type virus also suggests that contact ducks were exposed to a larger inoculum of H241Q virus.

Our results demonstrate that the pH of activation of the HA protein plays a key role in the pathogenicity and transmissibility of H5N1 influenza viruses in mallards. Natural H5N1 virus isolates are highly pathogenic in many, but not all, duck species (21, 47, 48), and their transmission among wild ducks and from wild ducks to domestic poultry and mammals, including humans, has been a key element in their natural ecology (10, 33, 54). Moreover, wild ducks are thought to be a main reservoir of low-pathogenicity avian influenza viruses (33). The intraspecies and interspecies transmission of influenza viruses depends on at least four factors: (1) the amount of virus shed by the donor; (2) the stability of the virus in the environment over time; (3) the time between donor shedding and acceptor exposure; and (4) the infectivity of the virus in the acceptor animal. As the pH of activation of the HA protein was found here to determine both the amount of shedding from ducks and the stability of virus in the environment, this molecular property may have an essential role in propagation of H5N1 viruses in aquatic birds. Furthermore, HA mutations that maximize virus shedding and environmental stability via altered HA acid stability may be expected to promote both intraspecies and interspecies transmission. A broad survey of the environmental stability of twelve low-pathogenicity avian influenza viruses of varying subtypes revealed that they were generally most stable at a slightly
basic pH (7.4-8.2), low temperature, and fresh to brackish salinity (1). The viruses lost infectivity much more rapidly after incubation in acidic conditions (pH < 6.6), warmer temperatures, and higher salinity. Among the HA mutations characterized in the present study, the N114K mutation increased the pH of activation to 6.4 while significantly reducing environmental stability, and the H24Q and K58I mutations reduced the pH of activation to 5.6 and 5.4, respectively, and moderately increased environmental stability. Thus, the pH of activation of the HA protein contributes to the duration of H5N1 influenza virus infectivity in the environment.

In the present study, an optimal range in the pH of activation of the HA protein supported the propagation of H5N1 influenza viruses in ducks. The adaptation of other subtypes of influenza viruses to different host tissues and species has been found to involve selection of viruses with altered pH values for membrane fusion. A few passages of egg-grown recombinant X-31 influenza virus (H3N2 with the internal genes of A/PR/8/34) in mammalian MDCK and MDBK cells consistently resulted in HA protein mutations that increased the pH of HA-mediated membrane fusion from 5.2 to 5.6-5.8, and similar results were found after the passage of egg-grown A/Japan/305/57 (H2N2) virus in MDCK cells (25). The natural adaptation of H7N3 influenza viruses from wild ducks to turkeys coincided with two amino acid mutations in and near the HA2 stalk and a decrease in the pH of activation of the HA protein without a change in receptor binding (13). However, it is not known whether these mutations exclusively caused the reduction in the pH of membrane fusion, because the adaptation also resulted in a 23-amino-acid deletion in the NA stalk that reduced neuraminidase activity. We showed here that the absence of neuraminidase activity results in a lower pH of membrane fusion by the HA protein. Moreover, decreased neuraminidase activity in H3N2 influenza viruses has been shown to reduce virus entry (28, 32). In general, there may be a cooperative interaction between the neuraminidase activity of the NA protein and the fusogenicity of the HA protein. A functional
The balance between neuraminidase activity and the receptor binding activity of the HA protein is well known in many influenza virus subtypes (17, 20, 29, 30, 52).

In influenza viruses of the H3N2, H7N1 and H7N7 subtypes, an increase in the pH of activation of the HA protein results in resistance to high concentrations of amantadine (>0.1 mM), which raise the endosomal pH (6, 9, 18, 44). In a recombinant virus bearing the envelope glycoproteins of A/Netherlands/219/03 (H7N7), an HA-G232C mutation in the fusion peptide that reduced the pH of membrane fusion from 5.4 to 4.4 reduced in vitro replication by more than 2 log_{10} and increased the MLD_{50} by more than 3 log_{10} (18). Thus, in mammalian species there may also be an optimal range of HA protein activation pH that supports efficient virus replication, infection, and pathogenicity. As high- and low-pathogenicity influenza viruses differ in their tissue tropism in avian and mammalian species (24, 27), the optimum pH values at which their HA proteins are activated to support successful infection and transmission may differ according to the influenza virus and the host species. Future investigation of the biological properties of the recombinant viruses from the present study in mouse and ferret models may reveal whether changes in the pH of activation of the HA protein support the adaptation and transmission of H5N1 influenza viruses in mammalian species, which is a significant factor in the pandemic potential of these viruses.
ACKNOWLEDGMENTS

We thank Scott Krauss, Heather Forrest, David Carey and Sharon Lokey for technical support in the BSL3+ facility. We thank Robert Lamb for the pCAGGS plasmid. We thank Rebecca DuBois and Stephen White for helpful discussions on protein structure and Sharon Naron for editing the manuscript. We thank the Hartwell Center for Bioinformatics & Biotechnology at St. Jude Children’s Research Hospital for DNA sequencing.

This project was supported by the National Institute Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN266200700005C, and by the American Lebanese Syrian Associated Charities (ALSAC).
Table 1. Genetic stability of recombinant H5N1 influenza viruses containing HA protein mutations after serial passages in embryonated chicken eggs.

<table>
<thead>
<tr>
<th>P1 Virus</th>
<th>P1 (\Delta pH^a)</th>
<th>P5 Mutations(^b)</th>
<th>P10 Mutations(^b)</th>
<th>P10 (\Delta pH^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y231H</td>
<td>+ 0.4</td>
<td>-</td>
<td>R228I + 0.4</td>
<td>-</td>
</tr>
<tr>
<td>H241Q</td>
<td>- 0.3</td>
<td>-</td>
<td>-</td>
<td>- 0.3</td>
</tr>
<tr>
<td>K582I</td>
<td>- 0.5</td>
<td>-</td>
<td>-</td>
<td>- 0.5</td>
</tr>
<tr>
<td>N1143K</td>
<td>+ 0.5</td>
<td>K1143N</td>
<td>K1143N</td>
<td>+ 0.2</td>
</tr>
</tbody>
</table>

\(^a\) Change in pH of membrane fusion (\(\Delta pH\)) relative to that of wild-type virus as measured by syncytium formation assay.

\(^b\) Dash indicates no change in HA protein sequence compared to the P1 virus. All of the mutations reported are in the HA gene (H5 numbering), and there were no amino acid sequence changes in the other genes.
Table 2. Environmental stability of H5N1 influenza viruses in water at 28 °C.

<table>
<thead>
<tr>
<th>Virus</th>
<th>LRM</th>
<th>R²</th>
<th>Estimated Persistence</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>6.8742 - 0.0974 x</td>
<td>0.7625</td>
<td>62 (10)</td>
</tr>
<tr>
<td>H241Q</td>
<td>6.9496 - 0.0775 x</td>
<td>0.8364</td>
<td>77 (13)</td>
</tr>
<tr>
<td>K58I2K</td>
<td>5.6297 - 0.4234 x</td>
<td>0.9680</td>
<td>14 (2)</td>
</tr>
<tr>
<td>K58H2I</td>
<td>6.6408 - 0.0761 x</td>
<td>0.7151</td>
<td>79 (13)</td>
</tr>
<tr>
<td>H241Q</td>
<td>6.1717 - 0.0991 x</td>
<td>0.7599</td>
<td>61 (10)</td>
</tr>
</tbody>
</table>

a LRM = linear regression model, \( y = \log_{10} \text{PFU/ml} \), \( x = \) persistence in days.
b Estimated days of virus persistence (starting virus titer of \( 1 \times 10^6 \) PFU/ml).
c Days required to reduce initial virus titer by \( 1 \log_{10} \).
Table 3. Morbidity and mortality caused by the recombinant H5N1 influenza viruses in mallards.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Infection Route</th>
<th>Death (No./Total)</th>
<th>Cloudy Eyes (No./Total)</th>
<th>Neurological Signsb (No./Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>Inoculated</td>
<td>4/6</td>
<td>5/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Contact</td>
<td>2/4</td>
<td>4/4</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td>Y231H</td>
<td>Inoculated</td>
<td>0/6</td>
<td>3/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Contact</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>H241Q</td>
<td>Inoculated</td>
<td>3/6</td>
<td>4/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Contact</td>
<td>4/4</td>
<td>2/4</td>
<td>2/4</td>
<td></td>
</tr>
<tr>
<td>K582I</td>
<td>Inoculated</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Contact</td>
<td>0/4</td>
<td>1/4</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>N1142K</td>
<td>Inoculated</td>
<td>0/6</td>
<td>2/6</td>
<td>2/6</td>
</tr>
<tr>
<td>Contact</td>
<td>3/4</td>
<td>2/4</td>
<td>2/4</td>
<td></td>
</tr>
</tbody>
</table>

Data are from two separate experiments. In each, 3 ducks were inoculated with 10^6 EID\textsubscript{50} of virus and 2 naïve contact birds were introduced into the cage 24 hours p.i. Birds were observed daily.

Twitching head, ataxia, violent tremors, severe torticolitis, and/or loss of balance.
Table 4. Tracheal and cloacal shedding of H5N1 influenza viruses by inoculated and contact ducks.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Day 3 p.i. No. shedding/Total</th>
<th>Day 5 p.i. No. shedding/Total</th>
<th>Day 7 p.i. No. shedding/Total</th>
<th>Day 10 p.i. No. shedding/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculated Trachea Cloaca</td>
<td>Contact Trachea Cloaca</td>
<td>Inoculated Trachea Cloaca</td>
<td>Contact Trachea Cloaca</td>
</tr>
<tr>
<td>wild-type</td>
<td>4(6)/2.5(4) 4(6)/3.1</td>
<td>3(4)/2.1</td>
<td>3(4)/2.1</td>
<td>2(4)/2.2</td>
</tr>
<tr>
<td>Y23,H</td>
<td>3(4)/1.9 4(4)/2.4</td>
<td>4(6)/1.9</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>K58,I</td>
<td>3(4)/1.1 1(4)/1.3</td>
<td>1(4)/1.3</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>N114,K</td>
<td>3(4)/3.2 2(4)/3.1</td>
<td>1(4)/1.8</td>
<td>1/4</td>
<td>1/4</td>
</tr>
</tbody>
</table>

* Number in parentheses denotes mean titer of shed virus in positive swabs (log_{10} EID_{50}/ml). Where swabs were positive but below the threshold of accurate measurement, a value of <1 log_{10} EID_{50}/ml was recorded.

Dash indicates that no animals survived.
Table 5. Titers (EID\textsubscript{50}) of H5N1 influenza viruses in the water dishes of mallards.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.63</td>
<td>3.25</td>
<td>1.50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Y231H</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H241Q</td>
<td>1.89</td>
<td>3.38</td>
<td>4.25</td>
<td>4.13</td>
<td>0</td>
</tr>
<tr>
<td>K582I</td>
<td>1.38</td>
<td>2.29</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N1143K</td>
<td>0</td>
<td>1</td>
<td>0.75</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are mean values from two separate experiments in which 3 ducks per group were inoculated with 10\textsuperscript{6} EID\textsubscript{50} of virus and 2 naïve contact birds were introduced into the cage 24 hours p.i.. Samples of drinking water (0.5 ml) were collected on days 1, 3, 5, 7 and 10 p.i. for virus titration in eggs.
<table>
<thead>
<tr>
<th>H5 numbering</th>
<th>H3 numbering</th>
<th>pH of membrane fusion</th>
<th>In vitro growth rate</th>
<th>Genetic stability</th>
<th>Estimated environmental persistence</th>
<th>Weight loss</th>
<th>Mortality</th>
<th>Rank order of shedding</th>
<th>Days in water dishes</th>
</tr>
</thead>
<tbody>
<tr>
<td>N114;K</td>
<td>N114;K</td>
<td>6.4</td>
<td>+</td>
<td>No</td>
<td>14</td>
<td>-</td>
<td>0 %</td>
<td>4 (lowest)</td>
<td>None</td>
</tr>
<tr>
<td>Y23;H</td>
<td>Y17;H</td>
<td>6.3</td>
<td>+++</td>
<td>Yes</td>
<td>61</td>
<td>-</td>
<td>60 %</td>
<td>2</td>
<td>1.3,5</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td>5.9</td>
<td>+++</td>
<td>Yes</td>
<td>62</td>
<td>+</td>
<td>70 %</td>
<td>1 (highest)</td>
<td>1.3,5,7</td>
</tr>
<tr>
<td>H24;Q</td>
<td>H18;Q</td>
<td>5.6</td>
<td>+++</td>
<td>Yes</td>
<td>77</td>
<td>+</td>
<td>0 %</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>K58;I</td>
<td>K58;I</td>
<td>5.4</td>
<td>+++</td>
<td>Yes</td>
<td>79</td>
<td>-</td>
<td>0 %</td>
<td>3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Recombinant influenza viruses in the background of A/chicken/Vietnam/CS8/04 (H5N1).
* Mutation denoted by number in amino acid sequence of H5 HA protein. The “1” subscript refers to numbering in HA1 and the “2” subscript refers to numbering in HA2 after cleavage.
* Number of mutation converted to conventional H3 numbering scheme.
* The highest pH at which syncytia formation was observed in vitro in Vero cells.
* Multiple-step growth rate in MDCK cells after infection with an MOI of 0.01 PFU/cell. The + symbol denotes a peak titer of ~5 log_{10} PFU/ml and the +++ symbol denotes a peak titer of ~7 log_{10} PFU/ml. Detailed data is reported in Figure 1B.
* No genetic stability refers to reversion of sequence within 5 serial passages in the allantoic cavities of embryonated chicken eggs. Viruses reported as “Yes” had no mutations after 5 passages in eggs and no changes in the pH of membrane fusion after 10 passages in eggs.
* Estimated environmental persistence is the calculated number of days of virus persistence at 28 °C (starting virus titer = 1 x 10^6 PFU/ml).
* Weight loss in directly infected ducks. A “-” symbol denotes continuous weight gain during the 14-day experiment, whereas a “+” symbol denotes a loss of 15-20% starting weight over the course of the first 5 days of infection.
* Percent mortality calculated from a total of 6 directly infected ducks and 4 contact ducks. Data for N114;K is excluded because this virus reverted to the wild-type sequence during the experiment.
* Rank order of shedding of virus from tracheal and cloacal swabs taken from both directly infected and contact ducks. Data for N114;K is excluded because this virus reverted to the wild-type sequence during the experiment. Detailed data is included in Table 4.
The days on which virus was detected in water dishes housed with ducks. "None" refers to no detectable virus on days 1, 3, 5, 7 and 10.

Data for N114;K is excluded because this virus reverted to the wild-type sequence during the experiment. Detailed data is included in Table 2.
FIGURE LEGENDS

Figure 1. Replication kinetics of recombinant A/chicken/Vietnam/C58/04 (H5N1) influenza viruses in MDCK cells. (A) For single-step growth curves, cells were infected at an MOI of 3 PFU/cell with wild-type virus or viruses containing HA protein mutations Y231H, H241Q, K582I or N1142K. (B) For multiple-step growth curves, cells were infected with the recombinant viruses at an MOI of 0.01 PFU/cell. Supernatant was collected at the indicated times and virus was measured by plaque assay. Each point represents the mean ± SD from three experiments.

Figure 2. The pH of HA-mediated membrane fusion by wild-type and mutant H5N1 influenza viruses in Vero cells was measured at 0.1 pH increments and is expressed as the highest pH value at which syncytium formation was observed.

Figure 3. Weight change in mallards infected with mutant and wild-type H5N1 influenza viruses. (A) Groups of ducks were inoculated with 10^6 EID_{50} of recombinant virus and (B) contact ducks were introduced into each group’s cage 1 day p.i.. Ducks were weighed daily for 14 days. Data points and error bars represent the mean (± SD) weight change. Viruses indicated in the figure key are ordered by increasing weight loss.

Figure 4. Contribution of NA enzymatic activity to the pH of membrane fusion mediated by the HA protein. (A) Representative photomicrographs of syncytia showing the contribution of the NA protein to HA-mediated membrane fusion. The pH values are shown in the top-left of each micrograph. The arrows point to examples of syncytia. rgC58: reverse-genetics wild-type C58 strain of H5N1 influenza virus. (B) Mean neuraminidase activity as measured by a fluorescence-
based assay using MU-NANA as substrate. Error bars represent the SD from three independent
determinations. Oseltamivir carboxylate (4 µM) was used to inhibit the enzymatic activity of the
NA protein in panels A and B.
REFERENCES:


A

infected ducks

body weight (% day 0)

time (day post infection)

B

contact ducks

body weight (% day 0)

time (day post infection)
**A**

<table>
<thead>
<tr>
<th>pH</th>
<th>pCAGGS HA only</th>
<th>pCAGGS HA + NA</th>
<th>Oseltamivir:</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**B**

NA activity (pmol/100µg total protein)

- pH 5.4
- pH 5.5
- pH 5.6

**Legend:**
- pH 5.4
- pH 5.5
- pH 5.6