Infectivity of Pseudotyped Particles Pairing Hemagglutinin of Highly Pathogenic Avian Influenza a H5N1 Virus with Neuraminidases of The 2009 Pandemic H1N1 and a Seasonal H3N2

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Abstract

Reassortment of influenza viruses is capable of generating novel virus strains, the emergence of which may come to represent major public health issues. Hemagglutinin (HA) and neuraminidase (NA) are the two major glycoproteins of influenza virus. These play a vital role in both the viral life cycle and evasion of the host immune response. Thus, predicting HA and NA reassortment, and characterizing the biology of HA and NA and of a novel virus are important for the control and prevention of influenza infection.

The HAs and NAs of three simultaneously circulating influenza viruses, a highly pathogenic avian influenza (HPAI) H5N1, the 2009 pandemic H1N1, and a seasonal H3N2, were evaluated by the pseudotyped particle (pp) system. Although the three HAs and NAs showed significant variation in their amino acid (aa) sequence, reassortment successfully generated infectious viral particles. Influenza H5 was demonstrated to have the ability to reassort with NAs from both the 2009 pandemic H1N1 and seasonal H3N2 viruses, resulting in highly infectious virions in both cases. All HAs in pps and wild-type viruses were predominantly HA0. Of the NAs, roughly half of the total N2 was present as a tetramer, 09N1 predominantly existed as a dimer, and the NA of H5N1 was primarily monomeric. Thus, tetrameric, dimeric, and monomeric NAs were all functional and could fulfill their role in viral life cycle.

Keywords: HPAI A (H5N1); Highly pathogenic avian influenza virus of type A (H5N1); HA; Hemagglutinin; NA; Neuraminidase; pp; Pseudotyped particle

Introduction

Influenza A virus belongs to a genus in the family Orthomyxoviridae and causes regular epidemics and periodic pandemics [1]. These latter events include the catastrophic H1N1 Spanish influenza of 1918, which killed more than 50 million people worldwide [2], the H2N2 Asian flu of 1957, which caused more than 1 million deaths [3], and the H3N2 Hong Kong flu of 1968, which caused 0.5 million deaths [4, 5]. The influenza A virus genome is contained on eight negative-sense single-stranded RNA segments that encode eleven proteins (HA, NA, NP, M1, M2, NS1, NEP, PA, PB1, PB1-F2, PB2) and is subtype according to the 16 HA and nine NA envelop proteins [1]. Influenza A viruses have a number of natural reservoirs. Many of these—including swine avian, and human—have both -2,3 and -2,6 linkages on the luminal surface of their respiratory tract epithelial cells. These hosts may thus serve as ‘mixing vessels’ for the genesis of new viral types by co-infection and resultant genetic reassortment [3, 6]. Indeed, genetic studies of the 2009 pandemic H1N1 strain have suggested that this virus contained a unique combination of gene segments from both North American and Eurasian swine lineages. Of these, PB2, PA, PB1, HA, NP, and NS were derived from a North American avian-like (triple reassortment), while NA and M were derived from an Eurasian swine lineage [7, 8]. That is, the 2009 pandemic H1N1 represents a successful example of influenza virus reassorting both across continents geographically and genotypes biologically.

The highly pathogenic avian influenza virus (HPAI) H5N1 has been circulating in Eurasia for more than a decade and has spread to more than 60 countries. To date, there have been 486 human H5N1 infections, resulting in 287 fatalities, with recent deaths reported in China, Indonesia, Vietnam, and Egypt [9]. The occasional direct transmission of the virus to humans suggests the possibility of a pandemic if consistent human-to-human transmission is achieved, and its high lethality has raised considerable concern worldwide.

Simultaneously, seasonal influenza kills an estimated 36,000 people in the United States each year [10]. Seasonal influenza viruses emerge from overlapping epidemics in East and Southeast Asia and then spread around the globe [10]. In the past decade, H3N2 has tended to have a markedly higher prevalence than H1N1, H1N2, or influenza B [11]. An analysis of 13,000 samples of influenza A/H3N2 viruses collected across six continents from 2002 to 2007 by the WHO’s Global Influenza Surveillance Network showed that newly emerging strains of H3N2 appeared in East and Southeast Asian countries 6-9 months earlier than in other locations. The viruses generally reached Australia and New

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Zealand next, followed by North America and Europe. The new variants typically reached South America after an additional 6-9 months [11]. At present, only two of the 16 possible HA subtypes, H1 and H3, and two of the nine possible NA subtypes, N1 and N2, are circulating in man [1]. Thus, reassortment of influenza virus RNA segments is theoretically capable of producing additional novel viruses. The recent emergence of H5N1, H5N2, and H3N2 supports this hypothesis [12, 13]. Such novel viruses could represent a great challenge to humanity if they are both highly pathogenic and transmissible. Thus, the ability to forecast possible reassortments of simultaneously circulating influenza viruses, particularly of the genes encoding the two envelope proteins, will benefit the ongoing efforts to control and prevent influenza infection.

In this study, we selected an H5N1 (A/Anhui/1/2005, #DQ371928) virus to represent highly pathogenic avian influenza viruses, the 2009 pandemic H1N1 virus (A/California/05/2009, #FJ966952) to represent pandemic influenza viruses, and H3N2 (A/Brisbane/10/2007, #CY035022) to represent the predominant seasonal influenza virus. The HAs and NAs matching pattern of these viruses was evaluated by the pp method.

Materials and Methods

Cell culture

For pseudotyping, A549 targeting cells and 293T human embryonic kidney pseudoparticle-producer cells (ACTT Catalog Number: CRL-11268) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified essential medium (DMEM; Invitrogen, Carlsbad, CA), supplemented with or without 10% fetal bovine serum.

Construction of NA and HA expression plasmids

The cDNA fragments encoding full-length HA proteins from A/Brisbane/10/2007 (#CY035022), A/California/05/2009 (#FJ966952), and A/Anhui/1/2005 (#DQ371928) influenza viruses, and the full-length NA proteins from A/Brisbane/10/2007 (#CY039089), A/Anhui/1/2005 (#DQ371928), and A/Ohio/07/2009 (#FJ966954) influenza viruses were cloned as reported previously [14-18]. HAs and NAs from H5N1, 2009H1N1 and H3N2 were designated AH H5 and AH N1; 09H1 and 09N1 and H3 and N2, respectively. For detection of AH N1 and N2, a 6×His tag was added by PCR at the C-terminal.

Pps production and quantification

As reported previously [14-18], pps were produced by transfecting 293T cells with four plasmids: an HA expression plasmid, an NA expression plasmid, a Gag-Pol-encoding plasmid, and a cytomegalovirus (CMV)-green fluorescent protein (GFP) reporter plasmid. At 72 h post-transfection, pps were harvested by passage through a 0.45µm Durapore polyvinylidene fluoride (PVDF) membrane filter (Millipore Ireland, Cork, Ireland). For quantification, purified pps were treated with 0.24 U/mL DNase and RNase at 37°C for 1 h to eliminate any contaminating DNA and RNA and then frozen at -70°C to inactivate the DNase and RNase. The pps were then treated with proteinase K (Qiagen, Valencia, CA) at 40°C for 30 min to digest the envelope proteins and release CMV–GFP RNA. Proteinase K was then inactivated by heating to 100°C for 2 min.

CMV–GFP RNA present in each pp was quantified by real-time quantitative reverse-transcription (qRT)-PCR [14, 17] using the forward primer 5′-CCCGTGAGTCAAACCGCATAT-3′, the reverse primer 5′-GTGATGCGTGGTTTGGACATA-3′, and the probe 5′-FAM-CCACGCCCATTTGATG-NFQ-3′, where FAM is the fluorescent dye 6-carboxy-fluorescein and NFQ is a non-fluorescent quencher. Assays were carried out using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and a PrimeScript One Step RT–PCR Kit (Takara, Japan). Pps were normalized for RNA copy number prior to infectivity and hemagglutination assays.

Infectivity assay

PPs were assayed for infectivity as reported previously [14-18]. Briefly, pps (normalized for RNA copy number) were diluted 1:1 in 100µl DMEM. To infect cells, cell culture medium was removed and replaced with the diluted pp suspension. The plate was allowed to stand for 4 h and the pp suspension removed and replaced with DMEM supplemented with 10% fetal bovine serum. At 72 h post-infection, infected cells were rinsed twice with phosphate-buffered saline (PBS). The number of GFP reporter-positive cells was determined by fluorescence-activated cell sorting (BD FACSAria; BD Biosciences, Franklin Lakes, NJ).

Hemagglutination assay

Normalized pp samples were diluted serially in 1:2 steps in PBS in microplate wells (50µL/well). Aliquots (50µL; 1% suspension) of turkey red blood cells were added to each well and hemagglutination was scored 30 min later.

Western blot analysis of HA and NA incorporation

To evaluate HA and NA incorporation into pps, we pooled pps resulting from four repeat experiments of each combination and concentrated them by centrifugation (25,000 rpm). Non-normalized pps (30µL) were then mixed with 4x SDS Sample Buffer (Invitrogen), heated to 100°C for 5 min, and analyzed by Western blotting as described below.

For Western blot analysis, prepared pp samples in nonreducing condition were first resolved by electrophoresis on 12% NuPage gels (Invitrogen). Separated proteins were transferred to PVDF membranes (Pall Corp., Port Washington, NY) using a semi-dry transfer method. For detection of HA and NA, membranes were blocked with 5% (w/v) skimmed milk in TBS-T overnight at 4°C and then incubated with primary antibody: either anti-09H1 (provided by Sino Biological Inc, Beijing, China), anti-AH H5 (provided by Prof. Mifang Liang), H3 (produced in our laboratory), anti-09N1 (provided by AbMax Biotechnology Co., Ltd, Beijing, China), or anti-His (mouse monoclonal antibody from CWBIO, Beijing, China) for 1.5 h at room temperature. After three washes in PBS, membranes were incubated with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 20 min at room temperature and again washed three times in PBS. NA and HA were then visualized using a DAB Substrate Kit (Vector Laboratories).

Data analysis

Differences were evaluated using a two-tailed Fisher's exact test (SPSS, release 12.1; SPSS Inc., Chicago, IL) and considered statistically significant at P < 0.05.

Results

Primary sequence analysis of three HAs and three NAs

For purposes of standardization, we aligned the primary sequences of the HAs and the NAs using the HA and NA from the A/Brisbane/10/2007 strain as a standard. Residues identical to those in the
Hemagglutinin:

A/Brisbane/10/2007 strain were replaced by ""; the amino acids in red represent the cleavage site of the HA precursor, linking the functional HA1 and HA2 domains; gray boxes indicate potential glycosylation sites.

Of the 586 aa in HA, 325 (55.46%) and 328 (55.97%) differences were detected in the HAs of the 2009 and H5N1 strains compared to those of the A/Brisbane/10/2007 strain, respectively (Figure 1). H3, 09H1, and AH H5 possessed 12, 6, and 7 potential glycosylation sites, respectively. The amino acids positions at H3N2 190, 194 and 226 (numbered by clustal W method, labeled in yellow), which were reported to be responsible for loss of the ability to bind to RBC, were D, I, and Q, respectively.

Of the 480 aa in NA, 260 (54.28%) of those in 09NA and 248 (51.77%) of those in NA from H5N1 were different to those from the A/Brisbane/10/2007 strain. N2, 09N1 and AH N1 possessed 8, 6, and 3 potential glycosylation sites, respectively (Figure 1). Residue 151 of N2 of H3N2 (labeled in yellow), which has also been reported to be related to loss of agglutination, was D, not G.

AH H5 pairs 09N1 and N2 with high infectivity

To study the HA and NA matching pattern, we generated nine types of pp by combination of three HAs and three NAs. Infectivity of these was then evaluated at a ratio of 10,000 (10000 viral RNA copies/1 target cell). In the pps group harboring H3, the infectivities of H3+N2, H3+AH N1 and H3+09N1 was 2.7±1.5%, 0.7±0.3% and 0.4±0.3%, respectively; thus, the native combination, H3+N2, showed the highest infectivity in this group (Figure 2). In the pps group harboring AH H5, the infectivities of AH H5+AH N1, AH H5+09N1 and AH H5+N2 was 92.3±1.6%, 7.1±3.9%, and 22.5±6.2%, respectively (Figure 2). The infectivity of the AH H5 group was significantly greater than that of the H3 and 09H1 groups (Figure 2). Among the AH H5 group, the native combination (AH H5+AH N1) displayed the highest infectivity, while the second highest was the combination of AH H5+N2 (22.5±6.2%; Figure 2). Taken together, although the three HAs and three NAs showed significant variation in their aa sequence, they were able to reassort successfully and generate infectious viral particles.

Neuraminidase:

Notably, H5 was demonstrated to have the ability to reassort with NAs from both the 2009 pandemic H1N1 and seasonal H3N2 viruses.

Hemagglutination assay

To further characterize combination HAs, normalized pp samples generated from the nine combinations were subjected to hemagglutination assay. All pps were diluted serially in two-fold steps in this assay. The hemagglutination abilities (log2) of AH H5+AH N1, AH H5+09N1 and AH H5+N2 (all containing AH H5) were 5.5±2.1, 6.0±1.4, and 6.0±1.4, respectively (Figure 3A). The hemagglutination abilities (log2) of 09H1+09N1, 09H1+AH N1, and 09H1+N2 (all containing 09H1) were 6.5±2.1, 5.5±2.1, and 6.5±2.1, respectively (Figure 3A). Interestingly, pps containing H3 were not capable of agglutinating turkey red blood cells, even at increased viral titers. This is consistent with the many reports that A/Brisbane/10/2007-like H3N2 viruses fail to agglutinate both avian and human type O red blood cells [20] (Figure 3A).

Evaluation of HA incorporation into pps

To evaluate incorporation of HA into the various pps, we pooled...
Notably, AH H5, 09H1, and H3 showed predominantly HA0. HAs of HPAI H5N1 viruses possess multiple basic amino acids at the cleavage site (RERRRKKR), which are cleaved by proteases ubiquitous in a wide range of organisms. This, together with the fact that the pp harboring AH H5 did not require trypsin treatment for infectivity assay, suggests that the mature form of AH H5 should be dominant. However, our data suggested that the amounts of the mature forms of all HAs present were similar (Figure 3B).

Taken together, these data suggest that all HA proteins were able to be incorporated into all pseudotype combinations. Combined the infectivity data, the loss of red blood cell agglutination is likely not due to the expression and incorporation of H3. Thus, the difference in infectivity of the combinations was probably due to the biological peculiarity of HAs in viral entry process.

**Incorporation of NA into pps**

Pooled, concentrated and non-normalizedpps were subjected to Western blot analyses in nonreducing condition. NA levels in all combinations were consistent with the pp numbers quantified by real-time quantitative PCR (data not shown). As shown in Figure 4, the molecular mass and form were dramatically different between N2, 09N1, and AH N1. 09N1 in pps was present predominantly as a monomer and dimer, with molecular masses of ~60 and ~120 kDa, respectively. AH N1 in pps was predominantly monomeric, with a molecular mass of ~50 kDa. N2 was present in pps as a dimer, tetramer, and possibly also a trimer. Thus, all NAs incorporated into pps effectively. Notably, although NA is believed to be present as a tetramer in the influenza virion, our data suggested that the predominating forms of AH N1, 09N1, and N2 differed. Indeed, only N2 was present as a tetramer; this form constituted roughly half of the total N2 present.

**Discussion**

There are two major strategies for influenza viruses to adapt to survive in a new host. One is evolution at both the nucleotide and amino acid levels; however the rate of evolution of avian influenza viruses is low [1]. Indeed, in wild aquatic birds, influenza viruses seem to be in an evolutionary stasis, suggesting optimal adaptation of these viruses to their hosts [1]. In contrast, all gene segments of mammalian and land-based poultry viruses continue to accumulate amino acid substitutions [1]. The other strategy is reassortment, which may dramatically change the biological character of a virus [12, 13]. The 1957 pandemic H2N2, the 1968 pandemic H3N2, the H1N2 that emerged in humans in 2001, the HPAI H5N1, and the 2009 pandemic H1N1 pandemic virus all had segmented genomes.

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**Figure 2: Infectivity of normalized pps.** Infectivity is presented as the mean percentage cells infected±SD (n = 4). Blue bars: infectivity of pps comprising native combinations of HA and NA (H3+N2, 09H1+09N1, AH H5+AH N1).

**Figure 3: Hemagglutination activity and HA incorporation status of pps.** (A) Hemagglutination assay. Pps were diluted serially in twofold steps in a 96-well plate. Hemagglutination activity is expressed as the mean HA titer (log, HA units/50µL) of each pps (n = 4). (B) Western blot analysis of HA in pps. Pooled pps of four repeat experiments for each combination were concentrated to assess the incorporation of NA in pps in nonreducing condition. The wild-type viruses HPAI H5N1 (A/Ahui/1/2005), 2009 pandemic H1N1 (A/California/7/2009), and H3N2 (A/Brisbane/10/2007) were used as positive controls.

**Figure 4: NA incorporation analysis.** Pooled pps of four repeat experiments for each combination were concentrated to assess the incorporation of NA in pps in nonreducing condition. Cell lysates of 293T, transfected by N3his only, was used as positive control. Naive 293T cell lysate was used as a negative control (-).
H1N1 had all undergone complex reassortments. The current approach to forecasting and confirming reassortment is reverse genetics; this could also result in man-made highly pathogenic novel viruses. The pseudotyped viral particle system can be used to package an artificial virion using the gag-pol protein of lentiviruses or other retroviruses [14-18]. Recently, this technology has been used to make pps of many viruses, including hepatitis B and C viruses and vesicular stomatitis virus (VSV) [15, 21]. Use of the pp system has advanced research on neutralizing antibodies and viral glycoprotein biology; especially for viruses that are either highly pathogenic or difficult to isolate [14-18]. The pp system is obviously safe and is a convenient model to study the reassortment of the two most important influenza virus antigens, HA and NA. In this study, we demonstrated that HA derived from the HPAI H5N1 strain could reassort with all NAs to generate highly infectious viral particles. The consequences of the possible emergence of such a virus for global health are, to say the least, significant.

The two surface glycoproteins of influenza virus, HA and NA, perform clearly defined complementary roles in virus infection [1]. Viral HA is responsible for attachment of virus to sialic acid-containing glycoconjugates on susceptible cells [1]. NA is responsible for destroying these receptors by removing the terminal sialic acid moieties, to promote release of progeny virus, thereby aiding virus transmission [1]. HA and NA are not only the most important antigens, but are also critical for viral entry, uncoating, assembly, and release [1]. Thus, study of the biology of HA and NA is important for enhancing our understanding of influenza viruses. In this study, we selected H5N1 to represent highly pathogenic avian influenza A viruses, the 2009 H1N1 to represent pandemic influenza A viruses, and H3N2 to represent the prevalent seasonal influenza A virus. We demonstrated that HAS and NAs of HPAI H5N1, 2009 pandemic H1N1, and seasonal H3N2 were all capable of generating infectious viral particles. These data raise the possibility of such reassortments occurring in nature.

Recent changes in the receptor binding characteristics of human H3N2 viruses are evident from alterations in RBC agglutination and the reduced growth capacity of recent isolates, particularly in embryonated eggs. The E190D (A/Alchi51/92), L194I (A/Sydney/5/97) and V226I (A/Sydney/5/97) mutations were reported to be responsible for the loss of the viruses ability to bind RBC [22, 23]. Meanwhile, a recent report suggested that changes in the receptor binding characteristics of human H3N2 viruses were not due to antigenic changes in HA, but were the result of a mutation in aspartic acid 151 of NA, to glycine, asparagine, or alanine [20]. Our data were not consistent with those reports; thus, the reason for the inability of the H3-harboring pps to agglutinate RBCs remains to be determined.

The NA activity was assayed using an NA-STAR influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems) as we reported previously [14, 15, 24], the activity of NA derived from H3N2 is between the highest (NA from 2009H1N1) and the lowest (NA from HPAI H5N1) (data not shown). Based on our previous studies, the NA activity is dependent on the interaction between NA and different spousc HA [14], high NA activity facilitates the virus budding and blocks virus entry [24], high NA activity makes the virus sensitive to oseltamivir, the higher of the NA activity, the more inhibition on virus release and the more increasing on virus entry when the virus was treated by oseltamivir [15].

We did not show our data regarding the efficiency of HA and NA expression in pp-producer cells because we have previously reported [24] that all displayed equal expression efficiencies. Taken together with the fact that HA/NA was successfully incorporated into pps and the pp quantification data, it seems likely that variation in infectivity is due to a biological peculiarities of HA/NA. Further peculiarities, including their response to a particular neutralizing antibody and any changes in HA/NA biology among all of the combinations, will be the subject of a future report from our laboratory.

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