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COMMENTARY
Host-Microbe Biology

Influenza's Newest Trick

Jeffery K. Taubenberger[a]

[a]Viral Pathogenesis and Evolution Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA

ABSTRACT Influenza A viruses are important pathogens for humans and for many birds and mammals. Hemagglutinin and neuraminidase are the major surface proteins of this enveloped RNA virus. Hemagglutinin requires proteolytic cleavage for activation, but because the viral genome does not encode its own protease, an exogenous serine protease must be provided by host cells. A novel, neuraminidase-dependent mechanism for hemagglutinin activation was described, in which a thrombin-like protease allows an influenza A/H7N6 virus, isolated from a mallard duck, to replicate systemically and induce enhanced disease in avian and mammalian model animals and to replicate *in vitro* in the absence of trypsin. Thrombin-like protease activation required the N6 neuraminidase, but also required the presence of a thrombin-like cleavage motif in the H7 hemagglutinin. This novel example of neuraminidase-dependent hemagglutinin activation demonstrates the extraordinary evolutionary flexibility of influenza A viruses and is a fascinating example of epistasis between the hemagglutinin and neuraminidase genes.

KEYWORDS hemagglutinin, influenza, neuraminidase, pandemic, viral pathogenesis

Influenza A viruses are the viral equivalents to the shape-shifting trickster Loki in Norse mythology. They infect numerous avian and mammalian hosts, their segmented RNA genomes enable genetic reassortment, and their RNA-dependent RNA polymerase lacks the ability to proofread, leading to a high error rate. These characteristics lead to continuous evolution that underlies a nearly infinite variety of "tricks," including host switch events, novel pandemic emergences, escapes from pre-existing human population immunity ("antigenic drift"), development of antiviral drug resistance, and mutational development of enhanced pathogenicity. Critically, the phenotypic consequences of this amazing evolutionary flexibility are unpredictable, allowing for the possibility that there remain other evolutionary tricks that have yet to be discovered and may appear in the future. Only through careful observation and pathogenesis studies, like the one described in a recent *mBio* article (1), can novel phenotypic properties of influenza viruses be identified.

The largest natural reservoir of influenza A viruses (IAVs) is in wild aquatic avian species, mostly in the orders Anseriformes (e.g., ducks, geese, and swans) and Charadriiformes (e.g., gulls and shorebirds), but host switch events allow IAV to infect domestic Galliformes (e.g., chickens, quails, and turkeys) and a number of mammalian species, including horses, dogs, swine, and humans (2, 3). Mammalian host switch events may cause limited epizootics, but avian IAVs may also adapt to new hosts, resulting in continuous viral circulation, pandemics, or panzootics. IAVs are enveloped, negative-strand, segmented RNA viruses, and they express two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). In the wild bird reservoir, 16 HA and 9 NA subtypes circulate and are found in 144 possible combinations (for example, H5N1 or H7N9), but fewer subtype combinations are found in IAVs that have adapted to humans (only H1N1, H2N2, and H3N2) and other mammals. HA and NA have complementary functions, with HA allowing IAV to bind and enter target epithelial cells

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Address correspondence to taubenbergerj@niaid.nih.gov.

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along the respiratory tract of mammals or the gastrointestinal tract of waterfowl, while the NA cleaves newly formed, budding virions from these cells, facilitating viral release and spread. The HA receptor binding domain recognizes cellular glycoproteins terminating in a sialic (neuraminic) acid, and the NA is an enzyme that cleaves sialic acids from cellular glycoproteins. These "opposing" functions require a balancing act between sialic acid binding and cleavage.

Following HA receptor binding, the virus is internalized, and the acidic pH in the endosome results in a conformational change in HA that mediates fusion of the viral and endosomal membranes, allowing release of viral ribonucleoproteins (RNPs) into the cytoplasm for viral replication. The mature HA molecule is a trimer, with each monomer undergoing proteolytic cleavage prior to activation. IAV does not encode its own protease and therefore requires exogenous serine proteases (trypsin-like enzymes) which recognize a conserved monobasic motif found at the HA cleavage site for activation. In humans and other mammals, this protease is likely to be the tryptase Clara, produced by cells of the bronchiolar epithelium. Avian IAVs that have adapted to gallinaceous poultry and express H5 or H7 HA subtypes can occasionally acquire insertional mutations at the HA cleavage site, changing their protease recognition motif to a furin-like polybasic amino acid sequence. This polybasic insertion broadens protease specificity, allowing intracellular cleavage activation, and consequently, systemic viral replication in infected poultry. Highly pathogenic avian influenza (HPAI) are defined as H5 or H7 IAVs that either have a polybasic cleavage site as determined by sequence analysis or induce at least 75% mortality in chickens experimentally infected to assess the intravenous pathogenicity index (IVPI) (4). Despite the association of some HPAI viruses with severe human disease, the term "highly pathogenic" does not relate to disease in humans or mammals, but specifically to disease in birds, especially chickens. The Eurasian lineage of HPAI H5N1 that has circulated widely in the last 2 decades—causing high mortality in domestic poultry—has also caused human zoonotic infections, with a high case fatality rate (5), possibly because of human genetic susceptibility (6).

In the new study by Kwon et al. (1), a low-pathogenicity avian influenza (LPAI) H7N6 virus showed systemic replication similar to replication of some HPAI viruses, and it was isolated from multiple tissues of a dead mallard duck in 2007. Surprisingly, however, this virus did not possess a polybasic HA cleavage site, as would have been expected for an H7 subtype virus that caused systemic avian infection. Moreover, in experimental infections of chickens, ducks, and mice, the H7N6 virus demonstrated systemic replication, and it was also able to replicate *in vitro* in the absence of trypsin but did not meet the IVPI criteria to be classified as HPAI in chickens.

Investigation of the basis for this strain's systemic replication revealed a new influenza virus "trick": a novel neuraminidase-dependent mechanism for trypsin-independent HA cleavage activation. To investigate the molecular basis of the observed trypsin-independent LPAI H7N6 viral activation, Kwon et al. (1) constructed a series of isogenic viruses on the H7N6 backbone that differed only in encoding a representative of each of the nine different avian NA subtypes, demonstrating that only the N6-expressing viruses replicated efficiently *in vitro* without trypsin. To determine whether these observations were specific to the N6 NA of the original duck isolate, isogenic viruses expressing each of six different N6 gene segments from other avian IAV were constructed. All supported trypsin-independent replication *in vitro*, indicating that avian IAV N6 NAs play a key role in the trypsin-independent growth of the H7 virus.

With support for the possibility that the N6 NA played a key role in HA activation, Kwon et al. (1) asked whether there were also features of the specific H7 HA sequence itself that might be critical for N6-associated, trypsin-independent HA activation. Sequence analysis showed that a number of Eurasian lineage H7s (including the H7N6 duck virus studied here) had an HA cleavage motif that could be bound by thrombin, suggesting that a thrombin-like protease might activate the H7 HA. Interestingly, genetic modification adding the thrombin-like cleavage motif to an H1 HA of a chimeric IAV strain with an N6 NA resulted in trypsin-independent replication *in vitro* and

systemic replication in mice. Conversely, altering the thrombin recognition motif of the H7 HA cleavage site abolished systemic replication and trypsin-independent growth. In a further set of experiments, the authors created a series of genetically related viruses expressing the N6 NA while expressing different avian IAV HA subtypes (H1 to H12) modified to encode the thrombin cleavage motif. They were able to rescue six of these isogenic viruses, all of which replicated efficiently *in vitro* in the absence of trypsin. Together, these observations support the hypothesis that viruses encoding both an N6 NA and an HA (of at least six different subtypes) encoding a thrombin-like HA cleavage motif, are needed for trypsin-independent growth in culture and for systemic replication in mice.

This is, however, not the first time that an IAV strain has been found to use an NA-dependent mechanism for trypsin-independent HA activation and subsequent systemic replication. A mouse-adapted derivative of the first-ever isolated human IAV, the influenza A/WSN/1933 H1N1 virus (WSN), replicates *in vitro* in the absence of trypsin and also replicates systemically in mice (7, 8). Data suggest that a mutation in the NA of the WSN virus allows it to bind plasminogen, sequestering it on the cell surface so that it can be activated to become plasmin. Plasmin, a serine protease, then recognizes the conserved monobasic arginine motif at the HA cleavage site. Importantly, plasminogen activation is dependent on the N1 NA of WSN. Notably, this curious feature of the mouse-adapted WSN virus, making it pathogenic in mice, is not a feature seen in human H1N1 viruses, including the 1918 pandemic virus.

Finally, Kwon et al. (1) sought to provide more evidence for their N6-associated, trypsin-independent HA activation hypothesis. They evaluated the impact of several different protease inhibitors on viral growth and HA activation. A broad-spectrum serine protease inhibitor efficiently blocked the replication of WSN in the absence of trypsin but did not inhibit the replication of the H7N6 virus. This drug, aprotinin, inhibits trypsin, plasmin, and other serine proteases but for some reason, not thrombin. A thrombin-specific protease inhibitor, argatroban, did however significantly inhibit the replication of the H7N6 virus, further supporting the hypothesis that a thrombin-like HA cleavage is the mechanism for trypsin-independent growth *in vitro* and systemic replication in animals.

What is the significance of these observations? First, IAV strains continue to evolve novel genotypic changes that can have important phenotypic implications for IAVs of birds and mammals. The authors showed that an LPAI H7N6 virus without a polybasic HA cleavage site can nonetheless replicate systemically in both avian and mammalian hosts to cause enhanced pathogenicity. Second, Eurasian lineage avian H7N9 viruses have caused zoonotic human infections since 2013, resulting in more than 1,500 confirmed infections with over 600 deaths (9). These H7 avian IAVs have undergone frequent reassortment events with other cocirculating avian IAV strains, and it is theoretically possible that H7N6 viruses could emerge to cause zoonotic human infections in which this novel, N6-dependent HA cleavage activation could allow human infections of viruses that might replicate systemically and possibly induce infections with higher pathogenicity.

Influenza is and will remain an important human and animal health problem. Its broad avian and mammalian animal range means that IAV cannot be eradicated and that new epidemic, zoonotic, and pandemic IAVs will continue to emerge, each with their own unique and unpredictable genotypes and phenotypes. Only through continued basic pathogenesis studies like the one described here and the development of more broadly protective "universal" influenza vaccines, can we be better prepared to control the impact of this continual threat.

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