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The ability of pandemic influenza virus hemagglutinins to induce lower respiratory pathology is associated with decreased surfactant protein D binding

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ABSTRACT

Pandemic influenza viral infections have been associated with viral pneumonia. Chimeric influenza viruses with the hemagglutinin segment of the 1918, 1957, 1968, or 2009 pandemic influenza viruses in the context of a seasonal H1N1 influenza genome were constructed to analyze the role of hemagglutinin (HA) in pathogenesis and cell tropism in a mouse model. We also explored whether there was an association between the ability of lung surfactant protein D (SP-D) to bind to the HA and the ability of the corresponding chimeric virus to infect bronchiolar and alveolar epithelial cells of the lower respiratory tract. Viruses expressing the hemagglutinin of pandemic viruses were associated with significant pathology in the lower respiratory tract, including acute inflammation, and showed low binding activity for SP-D. In contrast, the virus expressing the HA of a seasonal influenza strain induced only mild disease with little lung pathology in infected mice and exhibited strong *in vitro* binding to SP-D.

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Introduction

Influenza A virus (IAV) in humans primarily causes an acute respiratory infection with inflammation of the upper respiratory tree and trachea. While in most cases pneumonic involvement is not clinically prominent, virus can be recovered from both the upper and lower respiratory tract. Under certain still poorly understood circumstances, infection can lead to the development of viral pneumonia, characterized by injury to the gas exchange tissue, often with severe clinical consequences (Taubenberger and Morens, 2008). Severe disease is more often associated with the emergence of novel influenza viruses that cause pandemics (Simonsen et al., 1998; Wright, Neumann, and Kawaoka, 2007). In the last 100 years, influenza pandemics occurred in 1918, 1957, 1968, and 2009, and were associated with varying levels of morbidity and excess mortality (Morens, Taubenberger, and Fauci, 2009). Postmortem examinations from patients dying during these pandemics have revealed striking similarities in the spectrum of pathological changes in the lower respiratory tract (Gill et al., 2010; Kuiken and Taubenberger, 2008; Morens, Taubenberger, and Fauci, 2008; Mulder and Hers, 1972; Taubenberger and Morens, 2008). One key pathological feature in fatal cases, also prominent in postmortem examination of lung sections from victims of the 2009 H1N1 pandemic, was severe lower respiratory tract pathological changes associated with widespread infection of bronchiolar and alveolar epithelial cells and alveolar macrophages (Gill et al., 2010).

IAV hemagglutinin (HA) and neuraminidase (NA) are viral surface glycoproteins that mediate receptor binding and virion release from the host cell, respectively. HA recognizes sialic acid bound to underlying sugars on host cell glycoproteins. IAV adapted to birds have an HA receptor binding specificity for SA-α2,3-galactose, while HA from IAV adapted to humans typically have higher specificity for SA-α2,6-galactose (Matrosovich et al., 2000; Matrosovich et al., 1997; Rogers and Paulson, 1983). This binding preference is believed to be an important determinant of IAV cell tropism within a host. Lectin histochemistry studies have demonstrated that the respiratory tract of humans bears both α2-6 and α2-3 linked sialic acids but that α2-3 sialic acids are more prevalent in the lower respiratory tract (Nicholls et al., 2007; Shinya et al., 2006). Although pandemic viruses display preference for α2-6 linked sialic acids, autopsy reports indicate that they still can cause severe alveolitis, a consequence of infection of the lower respiratory tract. Moreover, in a recent study, Qi et al. (2009)

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demonstrated that IAV expressing the 1918 HA possessing either an SA-α2,6-galactose specificity, an SA-α2,3-galactose specificity, or a mixed SA-α2,6-galactose/SA-α2,3-galactose specificity were lethal to mice, with similar pathology and cellular tropism. This suggests that IAV respiratory tract tropism both in humans and mice must be influenced by other features of HA proteins than their SA binding specificity.

It has been reported that mannose-containing glycans on the viral HA and NA proteins can be recognized by components of the innate immune system, such as, for instance, C-type lectins of the collectin family, including surface surfactant protein D (SP-D) (Crouch, 2000; Hartshorn, 2010; Hartshorn et al., 1994; Hartshorn et al., 2008; Hartshorn et al., 1997; Hartshorn et al., 2007; Hartshorn et al., 2000; LeVine et al., 2001; Reading et al., 1997). SP-D is constitutively expressed by alveolar type II pneumocytes and non-ciliated bronchiolar (Clara) cells in the lower respiratory tract where it is considered to represent a counterpart of IgA, thereby playing a key role in the innate immune system (Crouch, 2000; Litvack et al., 2010). SP-D, together with other proteins, is believed to establish a first line of defense against both bacterial and viral pathogens by binding to carbohydrate moieties on the pathogen surface (Hartshorn, 2010). Most interestingly, SP-D has previously been shown to bind to carbohydrate residues on some influenza A viruses, thereby inhibiting their HA binding activity, causing viral aggregation (Hartshorn et al., 2008), and eventually leading to pathogen inactivation through activation of neutrophil and macrophage activity (Crouch, 2000; Hartshorn et al., 1994). Furthermore, SP-D may enhance viral clearance by binding to the carbohydrate side chains of IAV, blocking access of the HA receptor binding domain to cell surface receptors, and thus, interfering with virus internalization by host cells (Hartshorn et al., 2008). The ability of SP-D to bind the glycan component of HA protein has been shown to require high mannose type II glycans.

Previous analysis of the glycosylation patterns of the HA proteins from representative viruses of the 1957 H2N2 pandemic (A/Singapore/1/1957), the 1968 H3N2 pandemic (A/Aichi/1/1968), and a close relative of the pandemic 1918 influenza virus (A/Swine/Iowa/1931) observed an absence of type II glycans (Hartshorn et al., 2008; Schwarz and Klenk, 1981; Vigerust et al., 2007). While the exact glycosylation pattern of the 1918 and 2009 pandemic IAV HA proteins has yet to be determined, the deduced potential glycosylation pattern based on amino acid sequence predicts a lack of type II glycans (Reid et al., 1999; Stevens et al., 2004; Wei et al., 2010), and we hypothesized that the 2009 pandemic HA and the HA proteins of other pandemic IAV would have low binding activity with SP-D that would likely be associated with an enhanced ability of these viruses to invade deeper into the lower lung and infect alveolar lining cells.

In the present study, we tested this hypothesis and show that the HA proteins from pandemic influenza viruses show low SP-D binding *in vitro* and that chimeric viruses expressing the 1918, 1957, 1968, or 2009 pandemic virus HA induced more severe disease in mice as compared to seasonal IAV with increased SP-D binding activity. In addition, we show that enhanced alveolar and bronchiolar epithelial cell tropism is associated with severe lower lung pathology and more significant activation of host inflammatory responses. Our results demonstrate that low SP-D binding is a common feature of the HA proteins from the past four influenza pandemics and that this is also associated with increased morbidity, mortality, lung pathology, and inflammatory responses in mice infected with an otherwise isogenic virus.

Results

Rescue of chimeric viruses containing the HA gene of pandemic IAVs

Using reverse genetics, a series of isogenic chimeric 1:7 influenza viruses that contained the same 7 genomic segments derived from A/New York/312/2001 (H1N1) [NY312], but different HA segments were constructed. The latter encoded the HA gene of the 1918, 1957, 1968, or 2009 pandemic IAV, termed 1918-HA, 1957-HA, 1968-HA, or 2009-HA, respectively. The parental NY312 virus was also rescued. All rescued 1:7 chimeric viruses replicated to titers of 10^6–10^7 PFU/ml in cell culture (Table 1). To address if the HA/NA mismatch of the HA gene from H2 (1957) and H3 (1968) viruses on the NY312 H1N1 background could influence replication of the chimeric viruses, we also tested the 1918-HA, the 1957-HA, and the 1968-HA chimeric viruses in the context of their cognate NA by rescuing 2:6 viruses containing 6 NY312 gene segments and the HA and NA gene segments of each of the pandemic viruses and measuring growth in MDCK cells *in vitro* and their pathogenicity *in vivo*. These studies demonstrated that the properties of the 1:7 HA and 2:6 HA + NA expressing chimeric viruses tested in this study were very similar (Table 1). Similarly, the properties of the 2009-HA virus were similar to the wild-type A/Mexico/4108/09 (H1N1) virus (data not shown).

Morbidity and mortality of pandemic HA expressing isogenic viruses in mice

To test whether the isogenic chimeric viruses expressing seasonal or pandemic HA could cause differential disease and pathology in mice, groups of five 8–10 week old BALB/c mice were intranasally inoculated with each virus (Table 1) at a dose of 2×10^5 PFU. As shown in Fig. 1A, mice infected with the NY312 parental virus showed only very little weight loss during the first three days post infection. Whereas there was an average weight loss of about 10% in these mice by day 4 p.i., all mice eventually recovered and none of the animals died. In contrast, mice inoculated with the four pandemic HA expressing chimeric viruses all exhibited rapid and significant weight loss beginning from day 1 post-infection (dpi) reaching a nadir at 3–4 dpi (Fig. 1A). Even though peak weight loss in mice infected with 1957-HA and 1968-HA was similar to the peak weight loss observed with mice infected with NY312, by 4 dpi, mice infected with 1957-HA

Table 1
Properties of viruses tested.

Virus	Subtype	MDCK titer (PFU/ml $\log_{10}$)	Surfactant D protein (SP-D) binding assays		MLD_{50} (PFU/ml $\log_{10}$)
			HAI[a]	IC50[b]	
1918-HA:NY312	H1N1	7.2	>2 μg	>120 ng	5.25
1957-HA:NY312	H2N1	6.7	>2 μg	>120 ng	5.54
1968-HA:NY312	H3N1	6.5	>2 μg	>120 ng	5.41
2009-HA:NY312	H1N1	6.1	>2 μg	>120 ng	>5.6
NY312	H1N1	7.0	62.5 ng	7 ng	>5.7
1918-HA,NA:NY312	H1N1	6.8	>2 μg	>120 ng	3.8
1957-HA,NA:NY312	H2N2	6.5	>2 μg	>120 ng	5.04
1968-HA,NA:NY312	H3N2	6.6	>2 μg	>120 ng	5.45

[a] Mass of human SP-D protein required to inhibit agglutination of turkey RBCs.
[b] Determined by plaque reduction assay.