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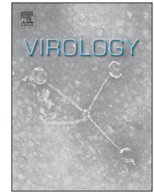

1918 Influenza receptor binding domain variants bind and replicate in primary human airway cells regardless of receptor specificity

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ABSTRACT

The 1918 influenza pandemic caused ~50 million deaths. Many questions remain regarding the origin, pathogenicity, and mechanisms of human adaptation of this virus. Avian-adapted influenza A viruses preferentially bind α2,3-linked sialic acids (Sia) while human-adapted viruses preferentially bind α2,6-linked Sia. A change in Sia preference from α2,3 to α2,6 is thought to be a requirement for human adaptation of avian influenza viruses. Autopsy data from 1918 cases, however, suggest that factors other than Sia preference played a role in viral binding and entry to human airway cells. Here, we evaluated binding and entry of five 1918 influenza receptor binding domain variants in a primary human airway cell model along with control avian and human influenza viruses. We observed that all five variants bound and entered cells efficiently and that Sia preference did not predict entry of influenza A virus to primary human airway cells evaluated in this model.

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1. Introduction

Influenza A viruses cause acute respiratory viral disease in humans in both annual epidemics and in infrequent pandemics (Wright and Kawaoka, 2007). The 1918 "Spanish" influenza pandemic resulted in approximately 50 million deaths globally and is the most severe influenza pandemic on record (Johnson and Mueller, 2002; Taubenberger and Morens, 2006). Many questions remain regarding the origin, pathogenicity, and mechanisms of human host adaptation of this deadly virus (Taubenberger and Kash, 2011). Influenza A viruses bind to terminal sialic acids (Sia) on target cell glycans and it is hypothesized that changes in the influenza A virus hemagglutinin (HA) protein receptor-binding domain (RBD) are important in the process of host adaptation, specifically allowing avian-origin influenza A viruses to adapt to humans.

The 1918 HA gene has been sequenced from multiple postmortem human lung samples, and several naturally occurring 1918 HA RBD sequence variants have been reported (Reid et al., 1999; Reid et al., 2003; Sheng et al., 2011). These include A/South Carolina/1/1918 (SC), which has an aspartic acid at both positions 187 and 222 in HA1 (H1 subtype numbering) (Reid et al., 1999) conferring an α2,6 Sia receptor-binding specificity and A/NY/1/1918 (NY), which differs from SC by a single amino acid, encoding a glycine at position 222 that confers a mixed α2,3/α2,6 binding specificity (Glaser et al., 2005; Stevens et al., 2006; Stevens et al., 2004). Sheng et al., 2011 reported two 1918 HA sequences with new RBD variants with yet to be confirmed binding specificities (Table 1). The HA RBD of A/Virginia/1/1918 (VA), which, in addition to aspartic acids at positions 187 and 222 has a change from glutamine to arginine at position 189 in the HA1 domain, may have an enhanced α2,6 binding specificity based on computational modeling (Sheng et al., 2011; Stevens et al., 2006). No binding specificity data is available for the A/New York/3/1918 (NY3), although deep sequencing revealed a predominance of asparagine rather than glycine at position 222 (Xiao et al., 2013). Finally, the 'avianized' laboratory-produced variant of the 1918 virus HA (AV), in which the aspartic acid at position 187 in NY was mutagenized back to the avian influenza virus consensus glycine, is reported to be exclusively α2,3 Sia binding (Glaser et al., 2005; Stevens et al. 2004, 2006).

It is not yet fully clear how influenza A virus Sia preferences, as predicted by *in vitro* glycan array analysis with limited numbers of

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Table 1
1918 Pandemic Influenza HA Receptor Binding Domain Variants Amino Acid Sequences

Virus (Reference)	Abbreviation	Residue at HA1 domain			Binding
		187	189	222	
A/South Carolina/1/1918 (Reid et al., 1999)	SC	D	Q	D	α2,6
A/New York/1/1918 (Reid et al., 1999)	NY	D	Q	G	α2,3 > α2,6
A/New York/3/1918 (Sheng et al., 2011)	NY3	D	Q	N	Unknown
A/Virginia/1/1918 (Sheng et al., 2011)	VA	D	R	D	α2,6 (modeled)
"Avianized" 1918 (Glaser et al., 2005; Stevens et al., 2006)	AV	E	Q	G	α2,3

synthetic oligosaccharides, relate to binding and entry of influenza viruses into the human respiratory tract, including the epithelium of the distal trachea and bronchi. Review of 1918 autopsy material, including correlative analyses of histopathology, distribution of influenza viral antigen by immunohistochemistry, and 1918 HA RBD variant gene sequencing, demonstrated no difference in cell tropism between the four naturally occurring 1918 RBD variants outlined above (Sheng et al., 2011). Autopsy sections demonstrated that the 1918 virus, regardless of HA RBD sequence, infected the entirety of the respiratory tract, including ciliated cells and goblet cells of the tracheobronchial tree and of the bronchiolar epithelium, and alveolar lining type I and type II cells. Based on lectin histochemistry, however, the upper airway and distal trachea in humans is reported to display predominantly α2,6 Sia on apical epithelial cell surfaces (Shinya et al., 2006; Nicholls et al., 2008; Davis et al., 2015). Mouse models of 1918 influenza viral infection also suggested that factors other than Sia preference play a role in influenza binding and entry to airway cells (Qi et al., 2009). Histopathological changes, cell tropism, and viral antigen distribution in lungs of mice infected with the 1918 RBD variants SC, NY, and AV were similar across viral variants and correlated with human autopsy findings.

Normal human bronchial epithelial (NHBE) cells, are primary human airway cells, and are variably reported to display exclusively α2,6 Sia or a mixture of α2,3/α2,6 Sia on their cell surfaces (Davis et al., 2015; Matrosovich et al., 2004; Ibricevic et al., 2006; Chan et al., 2010; Chan et al., 2013; Kogure et al., 2006). This laboratory has previously characterized the Sia distribution on a single lot of NHBE cells harvested from a healthy female donor and showed that this lot displays near exclusively α2,6 Sia on goblet and ciliated cell surfaces and rarely displays α2,3 Sia on goblet cell surfaces (Davis et al., 2015). Additionally it was shown that this lot of cells readily supports both human- and avian-adapted influenza virus growth to peak titers of 10^4-to-10^6 viral plaque forming units (pfu)/mL (Davis et al., 2015; Qi et al., 2014)., Experiments described here were conducted to evaluate the binding, entry, and peak replication of 1918 HA RBD variants, utilizing this lot of well-characterized NHBE cells, and to compare their binding and entry to human-adapted and avian H1 subtype influenza virus controls. Prevailing hypotheses suggest that variants with α2,6 Sia preferences would bind and enter human airway epithelial cells more efficiently than those the α2,3 Sia or mixed α2,3/α2,6 Sia preferences. Here NHBE cells were infected at a constant multiplicity of infection (MOI). In multiple experiments binding and entry was examined by immunofluorescence at 5-and 20-min post-addition of virus and quantification of cell-associated virus was performed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Viral replication was assessed at 12, 24, and 36-h post-infection in a subset of viruses representing the a range of Sia binding preference (AV α2,3; NY mixed α2,3 and 2,6; and SC α2,6).

2. Materials and methods

2.1. Growth and differentiation of cells

Primary Normal Human Bronchial/Tracheal Epithelial (NHBE) cells (CC-2541, Lonza; Walkersville, MD) from a single donor were grown as per manufacturer's instructions as described in detail previously (Davis et al., 2015). Briefly, NHBE cells were grown submerged in vendor-supplied medium on transwell-clear membrane supports coated with rat-tail collagen until fully confluent, at which point the apical medium was removed, creating an air-liquid interface and the media type was changed. Cells were then grown until they formed a mature pseudostratified epithelium, ~28 days total time.

2.2. Construction and rescue of chimeric viruses

Five variants of 1918 influenza A HA RBD virus were generated including four previously reported and a fully avianized version (Reid et al., 1999; Sheng et al., 2011; Glaser et al., 2005). Table 1 shows the amino acid sequences of each HA RBD variant's critical amino acid mutations, its hemagglutinin Sia binding preference (if known), and the abbreviated name as used in this study. In summary, all viruses but AV encode an aspartic acid (D) at position 187 [H1 numbering used throughout]; AV was engineered to encode the avian H1 influenza virus consensus glutamic acid (E) at this position. At position 222, SC and VA have D, NY and AV have glycine (G), and the HA of NY3 encodes an asparagine (N) (Sheng et al., 2011). VA encodes a D at positions 187 and 222 as does SC but additionally encodes an arginine (R) at position 189 instead of the consensus glutamine (Q).

The fully reconstructed 1918 H1N1 influenza viruses were isogenic except for the above HA RBD polymorphisms, and were prepared by reverse genetics as previously described (Qi et al., 2009). RBD mutations in the 1918 HA gene for each of the other viruses were generated with a site-directed mutagenesis kit following the manufacturer's instructions (Stratagene; La Jolla, CA). All rescued viruses were propagated in Madin–Darby canine kidney (MDCK) cells (ATCC; Manassas, VA). The genomic sequence of each rescued virus was then confirmed by sequence analysis of the inoculum prior to performing the experiments. All viruses and infectious samples were handled under BSL3+conditions in accordance with the Select Agent guidelines of the National Institutes of Health (NIH) and the Centers for Disease Control and Prevention under the supervision of the NIH Select Agent and Biosurety Programs and the NIH Department of Health and Safety.

2.3. Hemagglutination assay

Viral stocks were titered by plaque assay as previously described (Qi et al., 2009) and NHBE infections were normalized by multiplicity of infection (MOI). Given a primary experimental focus on viral binding and entry, viral stocks were also quantitated by

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